ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1985, p. 404–407
0066-4804/85/030404-04$02.00/0

Vol. 27, No. 3

Evidence for the Generation of Active Oxygen by Isoniazid Treatment of Extracts of *Mycobacterium tuberculosis* H37Ra

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Received 25 June 1984/Accepted 12 December 1984

Crude extracts of *Mycobacterium tuberculosis* H37Ra, an isonicotinic acid hydrazide (isoniazid) (INH)-susceptible strain which has peroxidase activity, catalyzed the production of catechol from phenol in the presence of INH and H_2O_2 as shown by the development of the 444-nm absorption peak of oxidized catechol product. Extracts of the INH-resistant strain of *M. tuberculosis* H37Ra, which has no peroxidase, did not catalyze the reaction. The rate of development of the 444-nm peak increased proportionately with increased superoxide dismutase concentrations. The hydroxyl radical (· OH) scavengers dimethylsulfoxide and mannitol inhibited the reaction. Isonicotinamide, isonicotinic acid, and nicotinic acid could not replace INH.

The oxidation of isonicotinic acid hydrazide (isoniazid) (INH) by peroxidase seems to contribute to its action (13, 18). INH oxidation intermediates appear to include peroxy, carbonyl, and isonicotinyl radicals (9, 14). The possibility of interconversion or side reactions of these radicals to yield other radicals, such as · OH and superoxide (O_2^-), is plausible. In a model system, horseradish peroxidase (HRP)-catalyzed oxidation of INH failed to produce · OH (14), although it produced a radical, probably a species equivalent to · OH such as a ferryl ion that could bleach *p*-nitrosodimethylaniline. Superoxide was produced only in the presence of an electron donor (15). Although HRP is quite similar to the peroxidase of *Mycobacterium tuberculosis*, they differ in some respects, such as the higher catalase activity and substrate specificity of the mycobacterial peroxidase (5). We have shown previously (14) that low concentrations of catalase accelerated the rate of nitroblue tetrazolium reduction by HRP. Thus, oxidation of INH by the mycobacterial peroxidase could differ in certain respects from HRP-catalyzed oxidation.

Hydroxyl radicals, O_2^-, and singlet oxygen ($^1O_2$) are toxic and mutagenic for cells (3, 6). Mycobacteria are susceptible to the catalase-H_2O_2-halide system (8), which is capable of producing active O_2. Also, it was proposed that the carotenoids of mycobacteria function primarily as scavengers of $^1O_2$, to which mycobacteria are suggested to be susceptible (4). In addition, mycobacteria are susceptible to high O_2 concentrations, and the MIC of INH against mycobacteria is lowered by increasing O_2 concentration (7). The uptake and metabolism of INH by mycobacteria are aerobic processes, and in the absence of O_2, INH has no effect. Since it seems that mycobacteria are susceptible to active O_2 and that O_2 plays a significant role in the mechanism of INH action, we examined the possibility of an INH-mediated active oxygen production in susceptible mycobacteria through oxidation of INH by endogenous peroxidase.

MATERIALS AND METHODS

Bacterial strains. *M. tuberculosis* H37Ra 201, which is INH susceptible, and 326, which is INH resistant, were obtained from the Mycobacterial Trudeau Culture Collection. Susceptibility or resistance to INH of both strains was confirmed by the method of the Center for Disease Control (1), and the MICs were found to be less than 1 μg for strain 201 and more than 5 μg for strain 326.

The catalase activity of each strain was confirmed by the column height technique and by the ability of extracts of each strain to decompose H_2O_2 by following the decrease in A_{240}. Peroxidase activity of these strains was confirmed by the ability of the extracts to oxidize *o*-dianisidine or 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (2) and by the ability of the cells to stain deep blue-black by treatment with a mixture of saturated benzidine solution and 3% H_2O_2.

Medium and growth conditions. Both strains were grown as pellicles on RV broth (10) without Tween 80 or bovine serum albumin.

Preparation of cell extracts. Pellicles (21 to 30 days old) of either strain were collected on a Buchner funnel with double-filter paper and washed extensively with distilled water. Three-gram quantities of the washed pellicles were transferred to sterile 250-ml flasks containing about 100 6-mm-diameter glass beads. Manual shaking of the pellicles with the glass beads for 2 min released both peroxidase and catalase into the soluble phase. Debris and unbroken cells were removed by suspending the resulting paste in a minimal amount of distilled water or phosphate buffer (pH 7) as required and centrifuging the pooled suspensions at 200 × *g* for 10 min. The supernatant fluid was decanted and used as cell extracts.

Partial purification of catalase and peroxidase of strain 201. Acidification of the cell extract of strain 201 by the dropwise addition of 2% acetic acid to pH 4.8 was found to precipitate most of the proteins in the crude extract other than the peroxidase and catalase proteins. Acidification of extracts that were prepared in distilled water was done while stirring gently and continuously with a small magnetic stir bar. The pH of the extract was continuously monitored during acidification with a glass pH electrode. If the pH was lowered to less than 4.5, both catalase and peroxidase activities were lost. Coagulated proteins were removed by centrifugation at 800 × *g* for 15 min. The supernatant fluid was collected, and the pH was adjusted to 4.8; the fluid was then recentrifuged and recollected.

The partially purified peroxidase preparations were then concentrated by vacuum dialysis to about 1/10 of their

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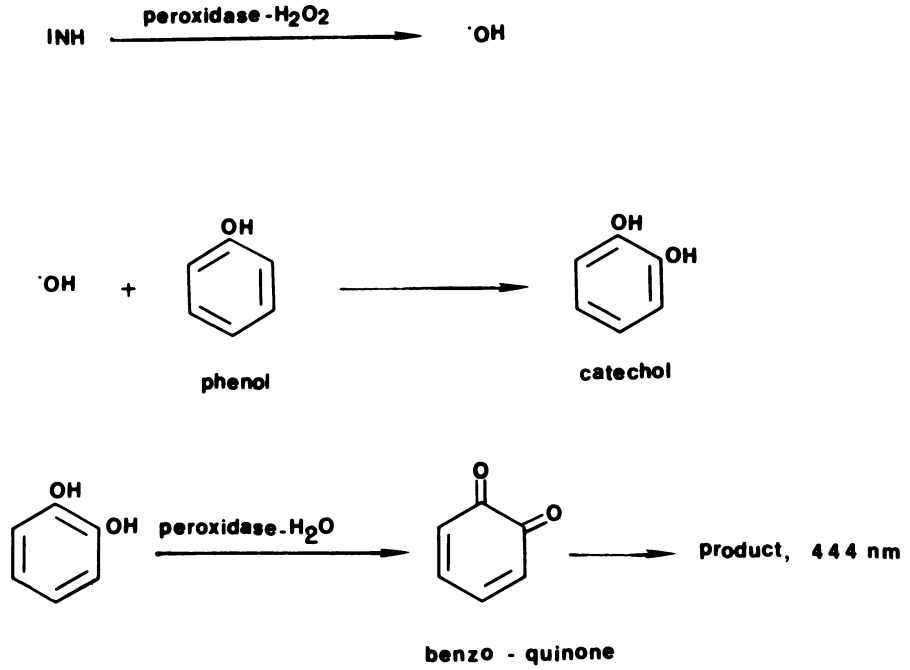

FIG. 1. INH-dependent · OH detection by sequential phenol hydroxylation and oxidation.

original volumes against 0.1 M phosphate buffer (pH 7.2). For this purpose, overnight dialysis through 10,000-dalton cut-off dialysis membranes was performed at 4°C.

Detection of · OH by phenol hydroxylation. In contrast to HRP, which can oxidize both phenol and catechol, the mycobacterial peroxidase could only oxidize catechol. Thus, the following method, which depends on phenol hydroxylation, can be applied only to the mycobacterial system. Phenol hydroxylation by · OH results in a mixture of hydroxylated products (11). One of the hydroxylation products is catechol, which can be oxidized by the mycobacterial peroxidase at the expense of H_2O_2. The chromophore absorbs at about 444 nm. The principle of this method is illustrated in Fig. 1.

The hydroxylation reaction was performed at 37°C in a final volume of 3 ml of 0.05 M phosphate buffer (pH 7.0) containing phenol, known amounts of the extracts of strain 201 or 326, and H_2O_2. These were incubated at 37°C for 7 min before starting the reaction by the addition of INH. For the purpose of scanning the spectrum of the reaction products, the reaction was allowed to proceed to completion (1 h) before scanning. The absorption maxima of the orange oxidation products were at 444 and 350 nm.

Protein measurement. The protein concentrations of the mycobacterial extract preparations were determined by the Bio-Rad reagent assay method with bovine liver catalase as a standard.

RESULTS

NBT could not be used for the detection of the generation of O_2^- by mycobacterial extracts in the presence of INH. Nitroblue tetrazolium was found to be reduced by cell extracts of both INH-susceptible and -resistant strains. This reduction was inhibited by superoxide dismutase (SOD). Cytochrome *c* (another O_2^- indicator) could not be used because of the ability of both INH and the cell extracts to reduce it. Indirect evidence for O_2^- production by INH-treated mycobacterial extracts was obtained during the study of · OH production.

Phenol hydroxylation experiments showed that extracts of the susceptible strain could mediate hydroxylation of phenol when INH and H_2O_2 were present. The spectra of the

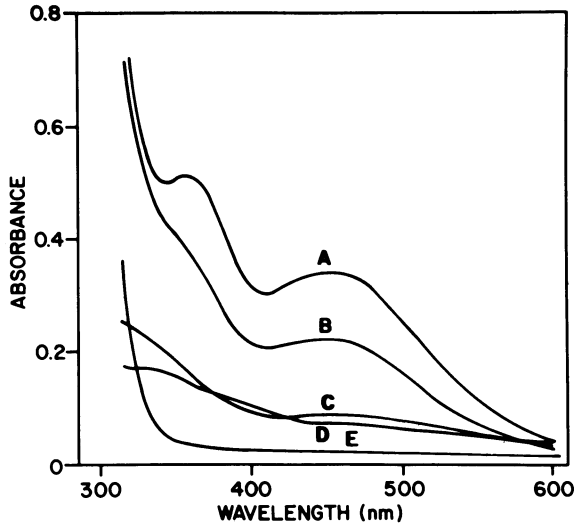

FIG. 2. Spectra of the hydroxylation products in the INH-treated extracts of INH-susceptible (peroxidase-positive) and -resistant (peroxidase-negative) strains of *M. tuberculosis* H37Ra. Reaction mixtures in 0.05 M phosphate buffer containing 1 mM H_2O_2 were incubated with the following at 37°C for 1 h in a final volume of 3 ml: (A) 30 μM catechol, 0.12 mg of susceptible strain extract, and 2.4 mM INH; (B) 13.1 mM phenol, 0.12 mg of susceptible strain extract, and 2.4 mM INH; (C) 13.1 mM phenol and 2.4 mM INH; (D) 13.1 mM phenol and 0.12 mg of susceptible strain extract; and (E) 13.1 mM phenol, 0.54 mg of resistant strain extract, and 2.4 mM INH. Reference cuvettes contained the same concentrations of both INH and extract used.

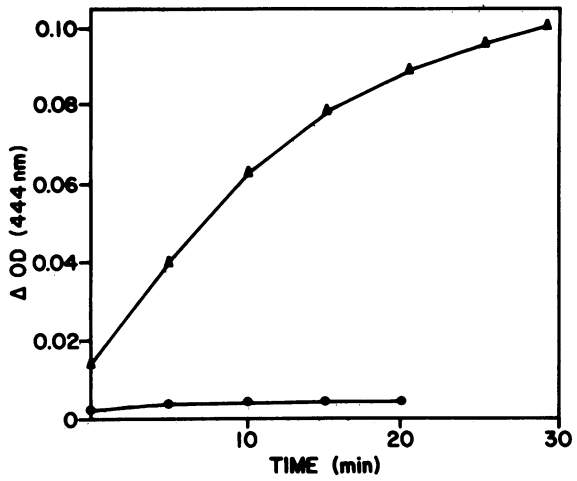

FIG. 3. Initial velocity of phenol hydroxylation induced by INH in extracts of susceptible and resistant strains of *M. tuberculosis* H37Ra. Reaction mixtures in 0.05 M phosphate buffer (pH 7.0) consisted of 0.12 mg of susceptible strain extract (▲) or 0.54 mg of resistant strain extract (●), 13.1 mM phenol, and 1 mM H_2O_2. They were incubated at 37°C for 7 min, 2.4 mM INH was added, and the change in A_{444} was recorded.

products in the complete reaction mixture containing phenol-INH-H_2O_2 plus susceptible strain extract gave the same spectrum as that of the control in which catechol replaced phenol (Fig. 2). The peaks of both reactions products were at 444 nm. Both spectra had a shoulder at about 350 nm. Replacing phenol by catechol and omitting INH resulted in the formation of a peak at 444 nm, but the shoulder at 350 nm was not formed. The resistant strain extract gave no absorption peaks in the spectrum range scanned. In the absence of INH, the susceptible strain extract also failed to produce the hydroxylation products or oxidized phenol peaks. Note that HRP can oxidize both phenol and catechol, whereas the mycobacterial peroxidase can oxidize catechol but not phenol.

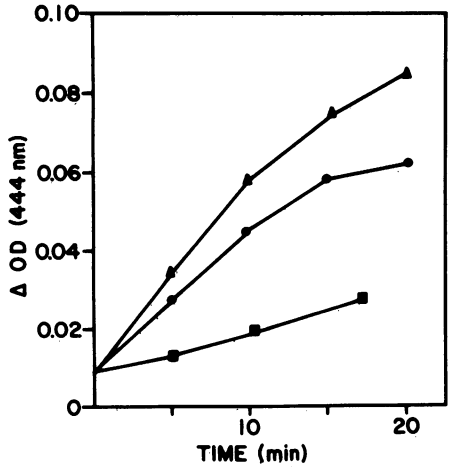

FIG. 4. Inhibition of INH-induced phenol hydroxylation by hydroxyl-radical scavengers. Reaction mixtures in 0.05 M phosphate buffer (pH 7.0) consisted of 0.12 mg of susceptible strain extract, 1 mM H_2O_2, 13.1 mM phenol, and 21 mM dimethyl sulfoxide (■), 40 μM mannitol (●), or buffer (▲). They were incubated at 37°C for 7 min, 2.4 mM INH was added, and the change in A_{444} was recorded.

The absorption peak at 444 nm was chosen for the study of the kinetics of the hydroxylation reaction. The susceptible strain extract mediated the formation of the hydroxylated products in the presence of INH, whereas the resistant strain extract did not (Fig. 3). The hydroxyl-radical scavengers mannitol and dimethyl sulfoxide reduced the rate of hydroxylation (Fig. 4). Dimethyl sulfoxide was effective at a concentration of 21 mM in reducing the hydroxylation rate by about 80%. Mannitol was less effective, reducing the rate about 30% (Fig. 4). Tris, another · OH scavenger, enhanced the hydroxylation reaction (unpublished data). As it appears from our HRP model system, alkaline pH enhances the reaction between INH and peroxidase (14). Thus, Tris-enhanced hydroxylations could be due to a pH effect caused by the strongly basic Tris.

The indirect evidence that O_2^- is also produced in the reaction was obtained from the fact that SOD enhanced the reaction (Fig. 5). The enhancement was directly proportional to the SOD concentration.

Other pyridine derivatives (isonicotinic acid, isonicotinamide, and nicotinic acid) failed to initiate hydroxylation to levels comparable to that of INH (Fig. 6). Hydrazine sulfate had no effect, and nicotinic acid hydrazide induced only a low rate of hydroxylation (data not shown).

DISCUSSION

The pathway of mycobacterial peroxidase-catalyzed oxidation of INH has not received as much attention as the oxidation products and their putative contribution to the mechanism of action of the drug. Winder (17) has speculated that free radicals are produced from INH through cupric ions or heme-catalyzed autooxidation. In previous studies (14), we have demonstrated evidence for a peroxidase-catalyzed INH oxidation pathway similar in some respects to the

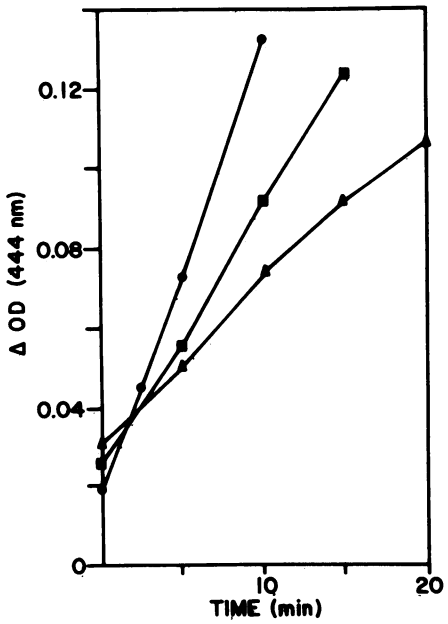

FIG. 5. Activation of INH-induced hydroxylation by SOD. Reaction mixtures in 0.05 M phosphate buffer (pH 7.0) consisted of 0.12 mg of susceptible strain extract, 1 mM H_2O_2, and 13.1 mM phenol with no (▲), 33 (■), or 67 (●) μg of SOD per ml. They were incubated at 37°C for 7 min, 2.4 mM INH was added, and the change in A_{444} was recorded.

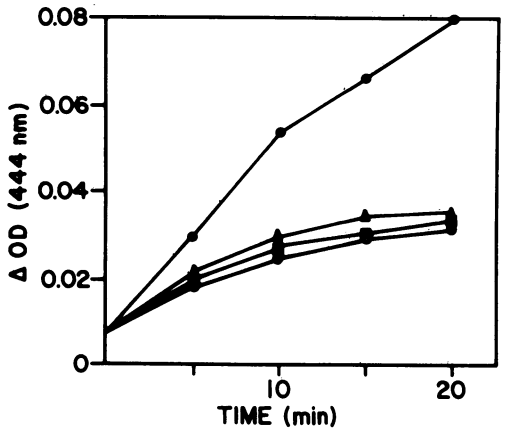

FIG. 6. Phenol hydroxylation by pyridine derivatives in susceptible strain extracts. Reaction mixtures in 0.05 M phosphate buffer (pH 7.0) consisted of 0.12 mg of susceptible strain extract, 1 mM H_2O_2, and 13.1 mM phenol. They were incubated at 37°C for 7 min, 2.4 mM of INH (●), isonicotinic acid (▲), isonicotinamide (■), or nicotinic acid (○) was added, and the change in A_{444} was recorded.

base-catalyzed autooxidation of INH in which free radicals might be involved. A contribution of the pyridine ring to the outcome of oxidation and the production of the proposed radicals was demonstrated. The peroxy radicals of INH (9) might under suitable conditions be able to interconvert into other radicals, such as · OH (16). Chances for interconversion are optimal in a complex biological system in which a wide variety of electron donors and acceptors as well as transition metal chelates are present. Thus, formation of · OH in the mycobacterial extracts is more likely to take place than in a pure chemical system in which peroxy radicals would have to proceed through isonicotinyl radicals that might easily lose their electrons by reacting with other oxidation intermediates.

In the present study, formation of a compound from phenol that absorbs at 444 nm is presumed to be an oxidized catechol product as shown by its matching spectrum with that of catechol oxidation product. The accelerating effect of SOD on the rate of formation of that compound is likely to be due to the supply of H_2O_2 from O_2^- which was formed during the reaction. Although the source of O_2^- is not known, it might be similar to that reaction of INH with HRP-H_2O_2 (compound I) in the presence of an electron donor (15). In the mycobacterial extracts, an electron donor might be an endogenous natural substrate for the peroxidase.

Further evidence that hydroxylation of phenol took place was through the inhibitory effect of dimethyl sulfoxide and mannitol. Thus, by lowering the rate of catechol formation, the rate of development of the peak at 444 nm decreased proportionately.

In the present study, external H_2O_2 was added to the reaction mixtures. No phenol hydroxylation took place in the absence of H_2O_2. Therefore, if · OH radicals are to form in intact cells, metabolic H_2O_2 formation in these cells should be a necessity. In this respect, the effect of INH on mycobacteria is reduced by reducing the metabolic rate in these cells by carbon source depletion (12). We suggest that this is due to the lowering of metabolic H_2O_2 formation, thus inhibiting the rate of the peroxidatical oxidation of INH by peroxidase. On the other hand, a direct measurement of H_2O_2 in mycobacteria is needed before one can attribute the effect to H_2O_2 formation.

The known role of peroxidase and O_2 in the mechanism of action of INH and the interpretation we give to our data suggest to us that · OH and O_2^- do contribute to the mechanism of action of INH.

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