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Enzymatic and Nonenzymatic Superoxide-Generating Reactions of Isoniazid

HUSSEIN A. SHOEB,[1] BERNARD U. BOWMAN, JR.,[1]* ABRAMO C. OTTOLENGHI,[1] AND A. JOHN MEROLA[2]

Department of Medical Microbiology and Immunology[1] and Department of Physiological Chemistry,[2] Ohio State University, Columbus, Ohio 43210

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During the course of horseradish peroxidase-mediated oxidation of either *o*-dianisidine or 2-2'-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS), no O_2 consumption took place. When isonicotinic acid hydrazide (isoniazid) (INH) was included in the reaction mixture, O_2 was consumed in amounts linearly related to the INH concentration. Nicotinic acid hydrazide at equimolar concentrations induced lower rates of O_2 consumption. Superoxide dismutase activated O_2 consumption. At equimolar concentrations, INH, nicotinic acid hydrazide, and phenylhydrazine induced bleaching of *p*-nitrosodimethylaniline in the horseradish peroxidase mediation of ABTS oxidation. Bleaching was not inhibited by hydroxyl radical (· OH) scavengers. After a short lag period, INH reacted with NADH at alkaline pH to produce superoxide radical (O_2^-), as detected by superoxide dismutase-inhibitable Nitro Blue Tetrazolium reduction. Nicotinic acid hydrazide with NADH caused a lower rate of O_2^- production after a longer lag period than INH.

The activity of the mycobacteriocidal drug isonicotinic acid hydrazide (isoniazid) (INH) has been linked to both mycobacterial peroxidase activity (3, 16) and NAD (11, 12) in several ways. The peroxidase enzyme of mycobacteria is thought to sensitize these cells to INH, and a strong positive correlation has been found (4). Sensitization of peroxidase-containing mycobacteria has been postulated to be through the oxidation of the drug with the production of toxic metabolites (8, 15). The correlation of NAD with INH susceptibility arose from the structural similarities between the drug and the nicotinamide moiety of NAD (12). In addition, NAD and NADP were reported to potentiate what is known as the Y-enzyme reaction (15). The enzyme catalyzes the production of a yellow pigment precursor (15) and was found to be the peroxidase in *Mycobacterium tuberculosis* H37Rv extracts (4). The precise role of both the peroxidase and NAD in the mechanism of action of INH is still obscure. In a recent study, we proposed that the oxidation of INH by peroxidase is by a free radical mechanism (13). These radical intermediates do not react with molecular oxygen efficiently at physiological pH (13). Assuming that in intact cells the peroxidase enzyme might act on natural phenolic and amino compounds that act as electron donors, we studied the effect of INH on the oxidation of some representative electron donors. The present study examined the possibility of superoxide (O_2^-) being produced by the peroxidase-catalyzed oxidation of both INH and an electron donor, as well as by the reaction of INH with NADH.

MATERIALS AND METHODS

O_2 consumption measurement. O_2 consumption by the reaction of INH with horseradish peroxidase (HRP) and an electron donor [2-2'-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS), *o*-dianisidine, L-epinephrine, or catechol] in the presence of H_2O_2 was measured in 0.01 M phosphate buffer (pH 6) as described previously (13). The rate of O_2 consumption at various INH concentrations was the same, whereas the total consumption increased linearly with increasing INH concentrations. Therefore, the values of this consumption were used for plotting dose-response curves. The order of addition of the reactants varied depending on the purpose of the experiment and is given in the figures.

***p*-NDA bleaching.** The measurement of bleaching of *p*-nitrosodimethylaniline (*p*-NDA) at 440 nm by the reaction of INH with HRP-H_2O_2-electron donor (ABTS) was carried out in 0.01 M phosphate buffer (pH 6.0) as described previously (13). Other pyridine and hydrazine derivatives (isonicotinic acid, nicotinic acid, isonicotinamide, nicotinic acid hydrazide, and phenylhydrazine) were also tested. In the absence of INH, HRP oxidizes ABTS at the expense of H_2O_2 to form a product that absorbs strongly at 414 nm (1). When INH was present in the reaction mixtures, the development of the 414-nm peaks was delayed for a time directly proportional to the INH concentration. Therefore, the time during which bleaching of *p*-NDA at 440 nm was recorded was selected to be within the lag period in the development of the 414-nm peak so as to avoid the overlapping of the variation in the two absorption peaks. When the other compounds tested had no effect on the oxidation reaction, the oxidation product of ABTS absorbed strongly at 440 nm, and *p*-NDA bleaching could not be followed.

Reduction of NBT. Nitro Blue Tetrazolium (NBT) reduction by the reaction of INH or nicotinic acid hydrazide with NADH was performed in 30 mM sodium bicarbonate (pH 9.5) as described previously (13). Superoxide dismutase (SOD) was added at various times.

Reagents. INH, nicotinic acid hydrazide, isonicotinic acid, isonicotinamide, HRP (type II), SOD, ABTS, L-epinephrine, *o*-dianisidine, and NBT were purchased from Sigma Chemical Co. Hydrogen peroxide (30% [vol/vol]) was purchased from Mallinckrodt, Inc., and *p*-NDA was purchased from Aldrich Chemical Co., Inc.

RESULTS

Induction of O_2 consumption by INH. The oxidation of *o*-dianisidine did not consume O_2 (Fig. 1). Oxygen evolution takes place as a result of the weak catalatic activity of the peroxidase. When INH was included in the reaction mix-

* Corresponding author.

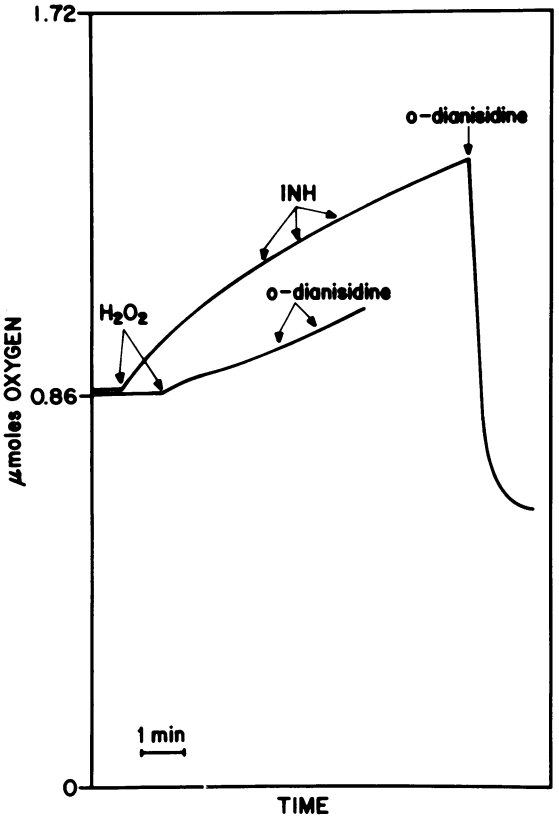

FIG. 1. Oxygen uptake induced by INH in the HRP–H_2O_2–*o*-dianisidine system. The reaction mixture at 37°C consisted of 25 μg of HRP and 100 μM *o*-dianisidine (10 μl from a methanolic solution) in 4 ml of 0.01 M phosphate buffer (pH 6.0). The sequence of addition of reactants was as indicated.

ture, the addition of *o*-dianisidine resulted in O_2 consumption. When ABTS was used instead of *o*-dianisidine, similar results were obtained. L-Epinephrine and catechol evoked minimal consumption of O_2 (data not shown). Under the conditions used, no change in the rate of O_2 consumption was observed at very low or very high concentrations of INH, but the total oxygen consumption increased with INH concentration (Fig. 2).

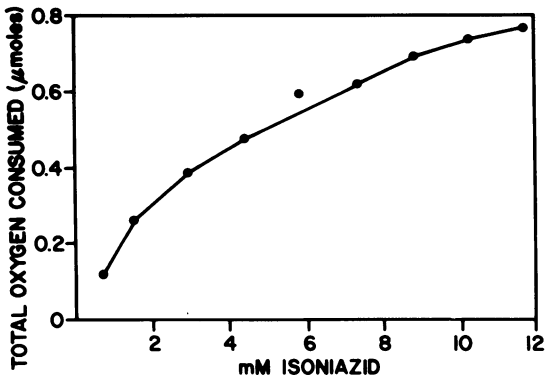

FIG. 2. INH-induced oxygen consumption in the HRP–H_2O_2–*o*-dianisidine system. The reaction mixture at 37°C consisted of 25 μg of HRP, 100 μM H_2O_2, and 1 mM *o*-dianisidine (10 μl of methanolic solution) in 4 ml of 0.01 M phosphate buffer (pH 6.0). The reaction was initiated by the addition of 1 to 12 mM INH to the reaction mixture as indicated.

Effect of SOD on INH-induced O_2 consumption. The generation of O_2^- in a reaction can be detected by the effect of SOD on the reaction, resulting in either inhibition or activation. In the present system, the reaction is H_2O_2 dependent, i.e., no O_2 consumption was observed in the absence of H_2O_2. Activation rather than inhibition resulted (Fig. 3) when SOD was included in the reaction of INH with HRP-H_2O_2-ABTS, possibly because of the production of H_2O_2 from O_2^- by dismutation according to the equation:

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2.$$

Thus, it is evident that O_2^- was produced in the system, resulting in an increased availability of H_2O_2 through dismutation. Control experiments in which each of the reactants was omitted one at a time showed that this O_2 consumption activation was not due to an independent SOD-catalyzed reaction with any of the reactants (data not shown).

O_2 consumption induced by nicotinic acid hydrazide. Nicotinic acid hydrazide (the meta isomer of INH) evoked O_2 consumption in the HRP-H_2O_2-ABTS system. The amounts of O_2 consumed by nicotinic acid hydrazide reactions ranged from 0 to 50% of the amounts consumed by INH reactions at equimolar concentrations of the isomers (Fig. 4).

***p*-NDA bleaching by INH and other related compounds.** We report elsewhere (13) that INH but not nicotinic acid hydrazide could bleach *p*-NDA in the HRP-H_2O_2 system. In the presence of an electron donor such as ABTS, the rate of *p*-NDA bleaching by INH is higher than that in the absence of the electron donor. Nicotinic acid hydrazide, which is

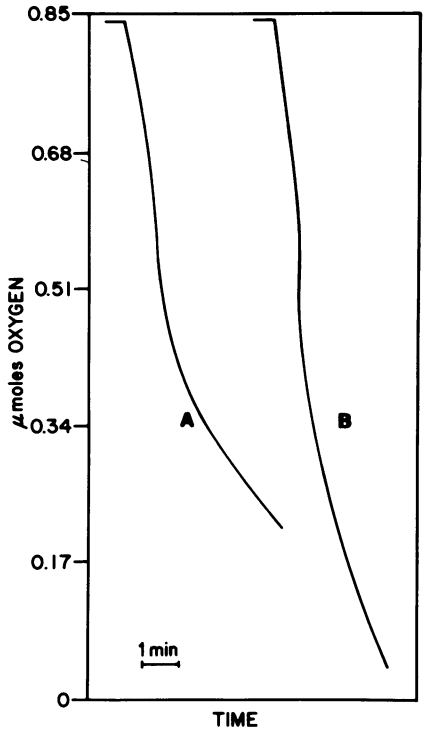

FIG. 3. Effect of SOD on the INH-induced oxygen uptake in the reaction of the HRP-H_2O_2-ABTS system. The reaction mixtures at 37°C consisted of 25 μg of HRP, 60 μM INH, 0.3 mM ABTS, and 0 (A) or 50 (B) μg of SOD per ml in 4 ml of 0.01 M phosphate buffer (pH 6.0). The reaction was initiated by the addition of 100 mM H_2O_2.

unable to bleach *p*-NDA in the absence of ABTS, induced *p*-NDA bleaching. Phenylhydrazine induced the highest rate of bleaching at equimolar concentrations, whereas isonicotinic acid, nicotinic acid, and isonicotinamide did not cause bleaching, and the absorption increase at 440 nm was due to the overlapping 414-nm peak of the ABTS oxidation product (Fig. 5) with the 440-nm peak. Bleaching of *p*-NDA by INH was linear with INH concentrations (Fig. 6) over a wide range. The hydroxyl radical scavengers mannitol and benzoate failed to inhibit *p*-NDA bleaching.

Reactions of INH with NADH. At neutral pH, no O_2^- production was detected from the reaction of INH with NADH. At alkaline pH, INH undergoes autooxidation and O_2^- is produced (9). At pH 9.5, the reaction of INH with NADH yielded O_2^- in high amounts, as shown by SOD-inhibited NBT reduction (Fig. 7). About 27% of that O_2^- was the result of autooxidation of INH at that pH (data not shown), as determined in the absence of NADH.

At equimolar concentrations, INH yielded much higher amounts of O_2^- than nicotinic acid hydrazide (Fig. 8). It is also shown that both INH and nicotinic acid hydrazide had a lag period, which was about 15 s in the case of the former and 2 min in the case of the latter.

DISCUSSION

The generation of O_2^- through the reaction of INH with peroxidase and NADH has been demonstrated in this report. The significance of O_2^- and O_2^--derived radicals in the mechanism of action of INH remains to be tested. However, several antibiotics and chemicals have been shown to exert their action through O_2^- (2, 5, 6), and the same might be relevant to INH action, although through a different pathway. Thus, although most O_2^--generating antibiotics have to be first reduced by reductases, INH seems to require at least partial oxidation by peroxidase or autooxidation at alkaline pH. Intermediates of oxidation in either case might then react with partially oxidized natural electron donors or NADH to give rise to O_2^-. Hydroxyl radicals could not be detected in the HRP system, and none would be likely to form, because the Haber-Weiss reaction (7) or iron-catalyzed Haber-Weiss-like reaction (10) would not take place since the H_2O_2 produced by O_2^- spontaneous dismutation would be destroyed efficiently by HRP.

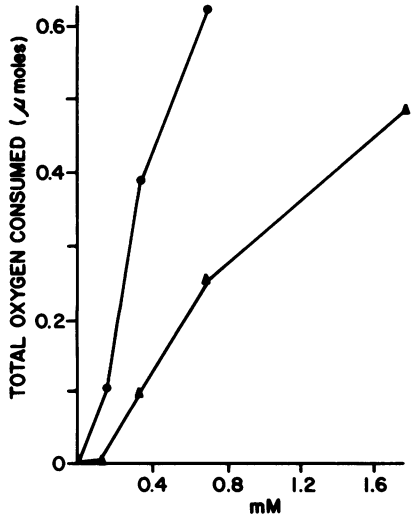

FIG. 4. Induction of oxygen uptake by INH or nicotinic acid hydrazide in the reaction with the HRP-H_2O_2-ABTS system. The reaction mixtures at 37°C consisted of 25 μg of HRP, 100 μM H_2O_2, and 0.3 mM ABTS in 4 ml of 0.01 M phosphate buffer (pH 6.0). The reaction was initiated by the addition of either INH (●) or nicotinic acid hydrazide (▲).

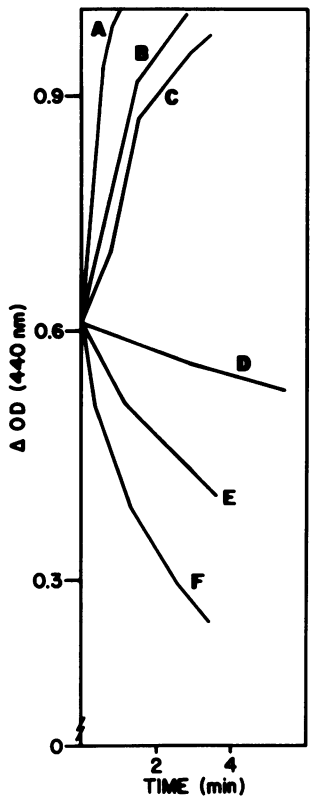

FIG. 5. *p*-NDA bleaching by pyridine and hydrazine derivatives by the reaction with the HRP-H_2O_2-ABTS system. The reaction mixtures at room temperature consisted of 0.2 mM *p*-NDA, 25 μg of HRP per ml, 100 μM H_2O_2, and 0.3 mM ABTS in 3 ml of 0.01 M phosphate buffer (pH 6.0). The reaction was initiated by the addition of 2.4 mM nicotinic acid (A), isonicotinic acid (B), isonicotinamide (C), nicotinic acid hydrazide (D), INH (E), or phenylhydrazine (F), and the change in A_{440} was recorded.

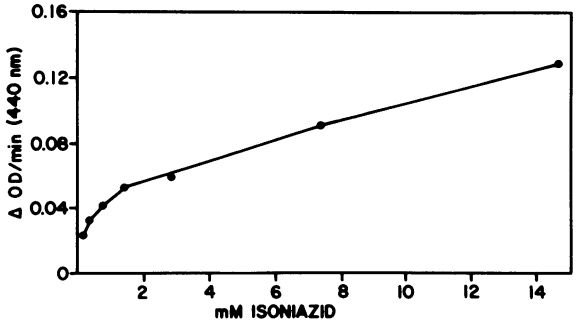

FIG. 6. *p*-NDA bleaching induced by INH in the HRP-H_2O_2-ABTS system. The reaction mixture at room temperature consisted of 0, 2, 4, 6, 8, 10, 12, or 14 mM INH, 100 μM H_2O_2, 0.2 mM *p*-NDA, and 0.3 mM ABTS in 3 ml of 0.01 M phosphate buffer (pH 6.0). The reaction was initiated by the addition of 1 μg of HRP. The reaction rates were calculated as the change in A_{440} per minute. Reference cuvettes contained the same concentrations of INH, HRP, and ABTS.

Phenylhydrazine might induce O_2^- production in the presence of HRP only. Phenylhydrazine is known to react with heme to produce O_2^- (6). Its spontaneous autooxidation is much faster than that of INH in the presence of a heme protein. That difference seems to stem from the contribution of the pyridine ring and the carbonyl group of INH, which contribute to its stability and requirement for high energy of activation. Autooxidation of phenylhydrazine in heme-free medium is also faster than that of INH. Comparison of INH, nicotinic acid hydrazide, and phenylhydrazine behavior in a medium containing *M. tuberculosis* cells could shed some light on the differences in their activity against the organism. Thus, at slightly acidic or neutral pH, phenylhydrazine would autooxidize so quickly that most O_2^- produced would be discharged externally to the organism. INH and nicotinic acid hydrazide undergo autooxidation very slowly, and they require an intracellular activation by the mycobacterial peroxidase. Once INH enters the cells and is acted on by the enzyme, it undergoes oxidation, yielding the radical intermediates described previously (13), whereas under similar conditions nicotinic acid hydrazide is only slowly oxidized due to the stability difference in its resonance structures (13). If these INH radical intermediates are the actual mechanism of killing, then INH would be expected to be more effective than nicotinic acid hydrazide and phenylhydrazine because (i) it can gain access to the interior of the cell in which it reaches vital structures and (ii) it undergoes activation by the peroxidase so that these intermediates are produced intracellularly in greater amounts than from nicotinic acid hydrazide.

The ability of nicotinic acid hydrazide to produce O_2^- in the presence of ABTS in the HRP system or in the alkaline reaction with NADH might indicate that the O_2^- produced resulted from the reaction of the partially oxidized hydrazine group with the intact or partially oxidized ABTS or with NADH. This also might apply to INH, since it undergoes the same three steps of oxidation to yield the diazine (9) moiety as does nicotinic acid hydrazide, although it follows a different scheme of oxidation after the carbanion step (9).

The production of O_2^- in INH treatment of mycobacteria has not been reported before. However, other evidence that this is likely comes from the fact that INH causes the accumulation of H_2O_2 upon treatment of cells (14). Hydrogen peroxide could result from O_2^- dismutation. As shown in the present model system, an electron donor is needed for O_2^- production, thus necessitating the presence of a natural electron donor in the mycobacterial cells to generate that radical. If our assumption is true, it would be interesting to study the role of O_2^- in the mechanism of INH action.

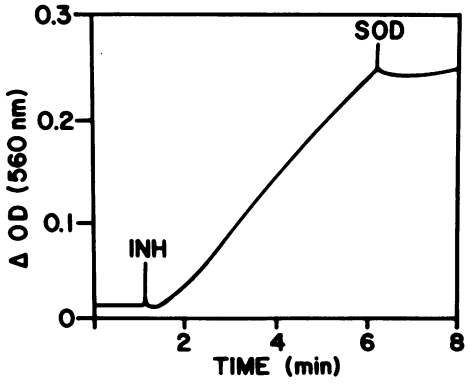

FIG. 7. SOD-inhibitable NBT reduction by the reaction of INH with NADH. The reaction mixture at room temperature consisted of 50 μM NADH and 0.2 mM NBT in 3 ml of 30 mM sodium bicarbonate (pH 9.5). The reaction was started by the addition of 2.4 mM INH at the time indicated, and the rate of formazan formation was recorded at 560 nm. At the time indicated, SOD was added to a final concentration of 7 μg/ml. The reference cuvette contained the same concentrations of both NADH and NBT in the same buffer.

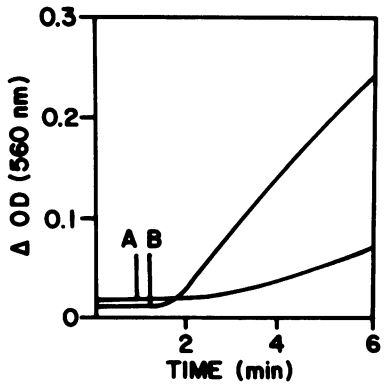

FIG. 8. Superoxide radical production by the reaction of NADH with either INH or nicotinic acid hydrazide. The reaction conditions were as indicated in Fig. 7. The reaction was started by the addition of 2.4 mM nicotinic acid hydrazide (A) or INH (B) at the indicated times.

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