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SUPEROXIDE DISMUTASE ENHANCES THE TOXICITY OF 3-HYDROXYANTHRANILIC ACID TO BACTERIA

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Cu,Zn·superoxide dismutase (SOD) enhanced the toxicity of 3-hydroxyanthranilic acid (3-HAT) to *Salmonella typhimurium* strain TA 102, evaluated as ability to form colonies. MnSOD showed the same effect. Inactivated Cu,ZnSOD had no effect. SODs accelerated the oxidation of 3-HAT, but inactivated Cu,ZnSOD caused little acceleration. It is proposed that the acceleration of 3-HAT oxidation leads to the enhancement of the 3-HAT toxicity. Catalase protected the bacteria from the toxicity of 3-HAT enhanced by Cu,ZnSOD, indicating that hydrogen peroxide generated in the oxidation of 3-HAT is involved in the toxixity. SODs accelerate the oxidation of 3-HAT and generate more hydrogen peroxide, that causes the enhancement of the 3-HAT toxicity to the bacteria. However, hydrogen peroxide alone was not so toxic. Hydrogen peroxide with 3-HAT was more toxic to the bacteria.

KEY WORDS: 3-Hydroxyanthranilic acid, oxidation, superoxide dismutase, *Salmonella typhimurium*, bactericidal effect, hydrogen peroxide

ABBREVIATIONS: SOD, superoxide dismutase; 3-HAT, 3-hydroxyanthranilic acid; BSA, bovine serum albumin

INTRODUCTION

Active oxygen species, such as the superoxide radical, hydrogen peroxide and the hydroxyl radical, have many deleterious effects.[1] They may be involved in cancer,[2,3] aging,[4] and inflammation.[5,6] Superoxide dismutase (SOD) removes the superoxide radical and thus protects against the toxicity of oxygen in biological systems.[1,7,8] SOD can prevent oxidative stress produced by daunorubicin[9] or by alloxan.[10,11] However, it was reported that SOD-rich bacteria were more readily killed by paraquat under aerobic conditions, possibly because of accumulation of more hydrogen peroxide.[12] Hence SOD may sometimes enhance oxygen toxicity.

3-Hydroxyanthranilic acid (3-HAT) is a tryptophan metabolite occurring in the kynurenine pathway, and is excreted in urine.[13] It is important as a precursor of NAD^+. On the other hand, 3-HAT has deleterious effects. 3-HAT is a known carcinogen, related to bladder cancer.[14–16] The mechanisms of carcinogenesis by 3-HAT are not fully elucidated, but its ability to oxidize may be relevant. 3-HAT oxidizes in a process that involves the production of organic and superoxide radicals, and that eventually yields cinnabarinic acid and hydrogen peroxide.[17–19]

$$3\text{-HAT} + O_2 \longrightarrow 3\text{-HAT}^{\cdot} + O_2^{\cdot-} + H^+ \quad \text{(a)}$$

$$3\text{-HAT}^{\cdot} + O_2 \longrightarrow \text{Quinoneimine} + O_2^{\cdot-} + H^+ \quad \text{(b)}$$

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad \text{(c)}$$

$$\text{Quinoneimine} + 3\text{-HAT} + 2O_2 \longrightarrow \text{Cinnabarinic acid} + 2H_2O_2 \quad \text{(d)}$$

The overall reaction is;

$$2(3\text{-HAT}) + 3O_2 \longrightarrow \text{Cinnabarinic acid} + 3H_2O_2 \quad \text{(e)}$$

In this paper we report the effects of SOD on the toxicity of 3-HAT to bacteria.

MATERIALS AND METHODS

Materials

3-Hydroxyanthranilic acid (3-HAT) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, U.S.A.). Nutrient broth and bacto-agar were obtained from Difco Laboratories (Detroit, Michigan, U.S.A.). Cu,ZnSOD was prepared from bovine erythrocytes and was assayed as described by McCord and Fridovich:[20] its activity was 3,600 U/mg. MnSOD from *Escherichia coli* was from Sigma Chemical Company (St. Louis, MO, U.S.A.) and was assayed in the same way:[20] its activity was 3,400 U/mg. For an inactive control, Cu,ZnSOD was exposed at pH 9.5, in 0.1 M sodium bicarbonate at 23°C, to 10 mM hydrogen peroxide for 1.5 h and was then thrice dialyzed against 500 volumes of the cold bicarbonate solution.[21] Bovine liver catalase from Boehringer Mannheim Biochemicals (Mannheim, West Germany) was dialyzed at 4°C against three changes of 500 volumes of 50 mM sodium phosphate buffer (pH 7.4) to remove preservatives. Its specific activity was found to be 38,000 U/mg protein. Protein concentration was determined according to Lowry *et al.*[22] and catalatic activity was by the methods of Beers and Sizer;[23] with 1 unit being able to decompose 1 μmole of H_2O_2 per min at pH 7.0 and 25°C when acting on 10.3 mM H_2O_2.

Bacteria

Salmonella typhimurium strain TA102 was kindly supplied by Dr. B. N. Ames (Berkeley, California, U.S.A.).[24] The bacteria were inoculated from frozen into 5 ml of nutrient broth media (0.8% nutrient broth, 0.5% NaCl) in 50 ml volume DeLong flasks. Inoculated cultures were grown aerobically on a rotary shaker at 200 rpm in the dark at 37°C for 14–16 h. After overnight growth the bacteria were harvested by centrifugation at 3,000 g for 10 min and washed thrice with 5 ml of 50 mM sodium phosphate buffer (pH 7.4). Finally, bacterial suspensions were prepared in the phosphate buffer. Concentration was adjusted to 0.50 OD at 600 nm in turbidity. The suspension contained 4.8×10^7 bacteria per ml, as determined by colony numbers on nutrient broth agar plates.

Exposure of the Bacteria to Reagents

3-HAT was dissolved to 0.2 mM in 50 mM sodium phosphate buffer (pH 7.4). The 3-HAT solution was passed through a Millex-GV filter (Millipore Corporation, Bedford, MA, U.S.A.) and used immediately.

40 μl of the bacterial suspension was added to 2 ml reaction mixtures in 13 mm diameter tubes. Exposure was carried out on a rotary shaker at 200 rpm at 37°C under air. After 0, 4, 8, and 24 h exposure, 50 μl aliquots were taken from the reaction mixtures and diluted by 10^4 with the phosphate buffer. Appropriate amounts (50 μl) of the dilutions were plated on nutrient broth agar (0.8% nutrient broth, 0.5% NaCl, 1.5% bacto-agar). The agar plates were incubated at 37°C for 24 h under air and colony numbers were counted. Some of the colonies were smaller than those of the control sample. These colonies were also counted. No new colonies emerged after 24 h incubation. Each sample was plated on two plates and colony numbers were averaged. Each experiment was replicated at least four times.

Oxidation of 3-HAT

Oxidation of 3-HAT was followed by increase in absorbance at 450 nm due to the oxidized product, cinnabarinic acid,[18] using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). The reaction was carried out in 13 mm diameter tubes on a rotary shaker at 200 rpm at 37°C under air. When bacteria were present in the reaction mixtures, they were removed through a Millex-GV filter just before the measurement of absorbance.

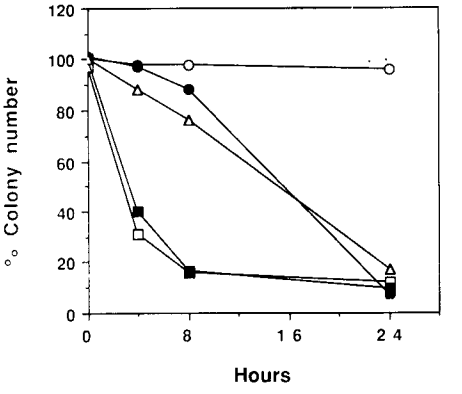

FIGURE 1 Exposure of the bacteria to 3-HAT and 3-HAT plus SODs. The bacterial suspension was added to reaction mixtures consisting of 50 mM sodium phosphate buffer (pH 7.4) and indicated reagents. Percent colony number denotes ratio to the colony number of the control sample at 0 h exposure. Values represent the average of independent experiments. (○), Control (n = 7); (●), 0.2 mM 3-HAT (n = 7); (□), 0.2 mM 3-HAT and Cu,ZnSOD (100 U/ml) (n = 7); (■), 0.2 mM 3-HAT and MnSOD (100 U/ml) (n = 4); (△), 0.2 mM 3-HAT and inactivated Cu,ZnSOD (corresponding to 100 U/ml) (n = 4).

RESULTS

Toxicity of 3-HAT is Enhanced by SOD

Salmonella typhimurium strain TA102 was exposed to 3-HAT (Figure 1). 3-HAT was toxic: it caused the bacteria to lose colony forming ability, while the bacteria unexposed to 3-HAT as a control maintained the ability almost completely during the 24 h. The toxic effect of 3-HAT was weak at 8 h exposure but prominent at 24 h exposure. Cu,ZnSOD markedly enhanced the toxic effect of 3-HAT (Figure 1). The colony number was markedly decreased at 4 h exposure to 3-HAT with Cu, ZnSOD and some of the colonies were smaller than those of the control, suggesting that some of the colony-forming bacteria has been damaged. After 8 h exposure the toxic effect of 3-HAT with Cu,ZnSOD was less marked. Cu,ZnSOD itself had no effects on the colony forming ability of the bacteria (data not shown). MnSOD showed almost the same effect as Cu,ZnSOD. Inactivated Cu,ZnSOD lost the toxicity-enhancing effect. These controls confirm that the enhancement of the 3-HAT toxicity by SODs is related to dismutation of the superoxide radical.

Oxidation of 3-HAT

Oxidation of 3-HAT was followed by increase in absorbance at 450 nm due to the oxidized product, cinnabarinic acid (Figure 2). The oxidation of 3-HAT was slow and continued after 24 h. Cu,ZnSOD greatly accelerated the oxidation, as previously reported.[25–27] The oxidation of 3-HAT with Cu,ZnSOD was completed in 8 h, when the toxicity of 3-HAT with Cu,ZnSOD reached almost the maximum effect (Figure 1). In the first 4 h about 90% of the oxidation was accomplished, when a strong toxic effect was observed. After 6 h the absorbance decreased gradually because of decay of cinnabarinic acid.[27] MnSOD had almost the same effect as Cu,ZnSOD. Inactivated Cu,ZnSOD had little accelerative effect: it may have accelerated the oxidation slightly because weak activity (0.8 U/ml) was left after the inactivation.

When bacteria were present in the reaction mixtures, they hardly affected the time courses of the oxidation of 3-HAT. But for the reaction mixtures containing 3-HAT and SODs, the absorbance at 450 nm was 15% higher with bacteria than without bacteria.

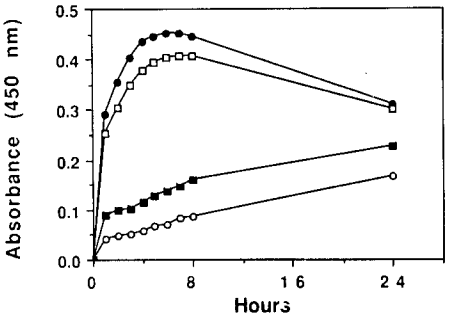

FIGURE 2 Oxidation of 3-HAT. Reaction mixtures contained 0.2 mM 3-HAT and indicated reagents in 50 mM sodium phosphate buffer (pH 7.4). (○), 3-HAT; (●), 3-HAT and Cu,ZnSOD (100 U/ml); (□), 3-HAT and MnSOD (100 U/ml); (■), 3-HAT and inactivated Cu,ZnSOD (corresponding to 100 U/ml).

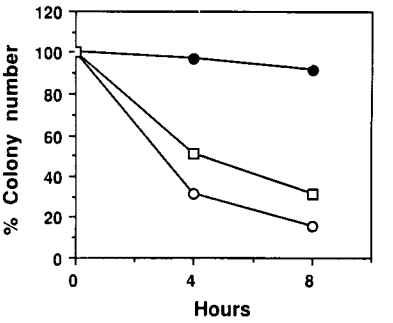

FIGURE 3 Effect of catalase on the toxicity of 3-HAT with Cu,ZnSOD. Procedures of the experiment were the same as in Figure 1. 3-HAT was 0.2 mM and Cu,ZnSOD was 100 U/ml. (○), 3-HAT and Cu,ZnSOD (n = 7); (●), 3-HAT, Cu,ZnSOD and catalase (26 μg/ml) (n = 7); (□), 3-HAT, Cu,ZnSOD, and BSA (26 μg/ml) (n = 5).

The time courses of the oxidation of 3-HAT were fairly parallel with the time courses of the toxic effects of 3-HAT on the bacteria (Figure 1 and 2).

Involvement of Hydrogen Peroxide

Hydrogen peroxide is generated in the oxidation of 3-HAT.[17-19] Catalase protected the bacteria from the toxicity of 3-HAT enhanced by Cu,ZnSOD (Figure 3). Bovine serum albumin (BSA) as a protein control for catalase caused little protection, indicating that the protection by catalase is not due to non-specific action of protein. Catalase and BSA themselves had no effects on the colony forming ability of the bacteria. The protection by catalase means that hydrogen peroxide is involved in the toxicity.

However, 0.3 mM hydrogen peroxide alone was less toxic to the bacteria (Figure 4). The toxicity of hydrogen peroxide was greatly enhanced by 3-HAT.

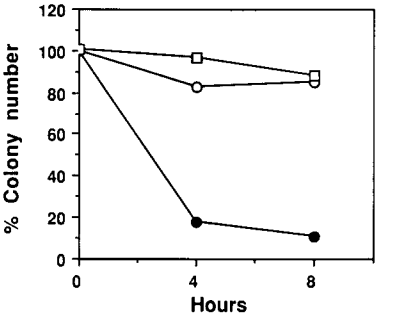

FIGURE 4 Exposure of the bacteria to H_2O_2. The bacterial suspension was added to reaction mixtures with or without 0.2 mM 3-HAT in the sodium phosphate buffer. Then a small aliquot of H_2O_2 was added to the reaction mixtures, to give a final concentration of 0.3 mM. The other procedures were the same as in Figure 1. (○), H_2O_2 (n = 5); (●) 3-HAT + H_2O_2 (n = 5); (□), 3-HAT (n = 5).

DISCUSSION

SOD increases the toxicity of 3-HAT to bacteria and catalase protects the bacteria from the toxicity, indicating that hydrogen peroxide is involved in the toxicity but the superoxide radical is not. SOD accelerates the oxidation of 3-HAT and will stimulate the generation of hydrogen peroxide. That explains the acceleration by SOD of the 3-HAT toxicity to the bacteria. The mechanisms of the acceleration by SOD of the 3-HAT oxidation are as follows.[26,27] SOD dismutes the superoxide radical in reaction (c), removing the superoxide radical in reactions (a) and (b). That shifts the equilibrium of reactions (a) and (b) to the right in favor of the formation of the superoxide radical, causing the acceleration of the 3-HAT oxidation and eventually stimulating the formation of hydrogen peroxide (e).

Hydrogen peroxide was involved in the toxicity, but equimolar hydrogen peroxide alone was not so toxic. Hydrogen peroxide with 3-HAT exhibited severer toxicity to the bacteria. Such behavior of hydrogen peroxide has been seen in a study of the bactericidal effect of hydrogen peroxide and ascorbic acid.[28] One possibility is that a strong toxicant is generated in the reaction of hydrogen peroxide and 3-HAT (or its oxidized intermediate). One candidate for such a strong toxicant is the hydroxyl radical, which can be generated from hydrogen peroxide and 3-HAT in the iron-catalyzed Haber-Weiss reaction.[26] However, involvement of the hydroxyl radical could not be determined. Dimethyl sulphoxide and ethanol, scavengers of the hydroxyl radical, and desferrioxamine, a chelator of iron,[29] showed some toxicity to the bacteria, so evaluation of their effects on the toxicity of 3-HAT was difficult. Another possibility is that hydrogen peroxide would help an intermediate of the 3-HAT oxidation to exert toxicity to the bacteria.

SOD enhanced toxicity of 3-HAT to the bacteria through the generation of more hydrogen peroxide. This implies that hydrogen peroxide is more hazardous than the superoxide radical under the conditions employed in our experiments. 3-HAT occurs in humans and has deleterious effects.[13–16] There is a possibility that SOD might aggravate the toxicity of 3-HAT to humans.

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References

1. J.V. Bannister, W.H. Bannister and G. Rotilio (1987) Aspects of the structure, function and applications of superoxide dismutase. *CRC Critical Reviews in Biochemistry*, **22**, 111–180.
2. R. Zimmerman and P. Cerutti (1984) Active oxygen acts as a promoter of transformation in mouse embryo C3H/10T1/2/C18 fibroblasts. *Proceedings of the National Academy of Science of the United States of America*, **81**, 2085–2087.
3. L.W. Oberley and G.R. Buettner (1979) Role of superoxide dismutase in cancer: a review. *Cancer Research*, **39**, 1141–1149.
4. B.E. Leibovitz and B.V. Siegel (1980) Aspects of free radical reactions in biological systems; aging. *Journal of Gerontology*, **35**, 45–56.
5. B.M. Babior, R.S. Kipnes and J.T. Curnutte (1973) Biological defence mechanisms; the production by leukocytes of superoxide, a potential bactericidal agent. *Journal of Clinical Investigation*, **52**, 741–744.

6. R.C. Allen, S.J. Yevich, R.W. Orth and R.H. Steele (1974) The superoxide anion and singlet molecular oxygen; their role in the microbicidal activity of the polymorphponuclear leucocyte. *Biochemical and Biophysical Research Communications*, **60,** 909–917.
7. N.M. Hassan and I. Fridovich (1977) Regulation of the synthesis of superoxide dismutase in *Escherichia coli*. *Journal of Biological Chemistry*, **252,** 7667–7672.
8. D.O. Natvig, K. Imlay, D. Touati and R.A. Hallewell (1987) Human copper-zinc superoxide dismutase complements superoxide dismutase-deficient *Escherichia coli* mutants. *Journal of Biological Chemistry*, **262,** 14697–14701.
9. J.W. Lown, S.-K. Sim, K.C. Majumdar and R.-Y. Chang (1977) Strand scission of DNA by bound adriamycin and daunorubicin in the presence of reducing agents. *Biochemical and Biophysical Research Communications*, **76,** 705–710.
10. K. Grankvist, S. Marklund, J. Sehlin and I.-B. Täljedal (1979) Superoxide dismutase, catalase and scavengers of hydroxyl radical protect against the toxic action of alloxan on pancreatic islet cells *in vitro*. *Biochemical Journal*, **182,** 17–25.
11. L.J. Fischer and S.A. Hamburger (1980) Inhibition of alloxan action in isolated pancreatic islets by superoxide dismutase, catalase, and a metal chelator. *Diabetes*, **29,** 213–216.
12. M.D. Scott, S.R. Meshnick and J.W. Eaton (1987) Superoxide dismutase-rich bacteria. *Journal of Biological Chemistry*, **262,** 3640–3645.
13. E. Boyland and D.C. Williams (1956) The metabolism of tryptophan. *Biochemical Journal*, **64,** 578–582.
14. E. Boyland and G. Watson (1956) 3-Hydroxyanthranilic acid, a carcinogen produced by endogenous metabolism. *Nature*, **177,** 837–838.
15. M.J. Allen, E. Boyland, C.E. Dukes, E.S. Horning and J.G. Watson (1957) Cancer of the urinary bladder induced in mice with metabolites or aromatic amines and tryptophan. *British Journal of Cancer*, **11,** 212–228.
16. G.T. Bryan, R.R. Brown and J.M. Price (1964) Mouse bladder carcinogenicity of certain tryptophan metabolites and other aromatic nitrogen compounds suspended in cholesterol. *Cancer Research*, **24,** 596–602.
17. G. Ehrensvärd, J. Liljekvist and R.G. Health (1960) Oxidation of 3-hydroxy-anthranilic acid by human serum. *Acta Chemica Scandinavica*, **14,** 2081–2088.
18. P.V. Subba Rao and C.S. Vaidyanathan (1966) Enzymic conversion of 3-hydroxyanthranilic acid into cinnabarinic acid. *Biochemical Journal*, **99,** 317–322.
19. H. Ogawa, Y. Nagamura and I. Ishiguro (1983) Cinnabarinate formation in malpighian tubules of the silkwork, *Bombxy mori:* reaction mechanism of cinnabarinate formation in the presence of catalase and manganes ions. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*, **364,** 1507–1518.
20. J.M. McCord and I. Fridovich (1969) Superoxide dismutase. *Journal of Biological Chemistry*, **244,** 6049–6055.
21. D.M. Blech and C.L. Jr. Borders (1983) Hydroperoxide anion, HO_2^-, is an affinity reagent for the inactivation of yeast Cu, Zn superoxide dismutase: modification of one histidine per subunit. *Archives of Biochemistry and Biophysics*, **224,** 579–586.
22. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193,** 265–275.
23. R.F. Jr. Beers and I.W. Sizer (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, **195,** 133–140.
24. D.E. Levin, M. Hollstein, M.F. Christman, E.A. Schwiers and B.N. Ames (1982) A new *Salmonella* tester strain (TA102) wit A·T base pairs at the site of mutation detects oxidative mutagens. *Proceedings of the National Academy of Science of the United States of America*, **79,** 7445–7449.
25. J.A. Dykens, S.G. Sullivan and A. Stern (1987) Oxidative reactivity of the tryptophan metabolites 3-hydroxyanthranilate, cinnabarinate, quinolinate and picolinate. *Biochemical Pharmacology*, **36,** 211–217.
26. H. Iwahashi, T. Ishii, R. Sugata and R. Kido (1988) Superoxide dismutase enhances the formation of hydroxyl radicals in the reaction of 3-hydroxyanthranilic acid with molecular oxygen. *Biochemical Journal*, **251,** 893–899.
27. T. Ishii, H. Iwahashi, R. Sugata, R. Kido and I. Fridovich (1990) Superoxide dismutases enhance the rate of autoxidation of 3-hydroxyanthranilic acid. *Archives of Biochemistry and Biophysics*, **276,** 248–250.
28. T.E. Miller (1969) Killing and lysis of gram-negative bacteria through the synergestic effect of hydrogen peroxide, ascorbic acid, and lysozyme. *Journal of Bacteriology*, **98,** 949–955.

29. J.M.C. Gutteridge, R. Richmond and B. Halliwell (1979) Inhibition of the iron-catalyzed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. *Biochemical Journal*, **184**, 469–472.

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