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Hydrogen Peroxide Kills *Staphylococcus aureus* by Reacting with Staphylococcal Iron to Form Hydroxyl Radical*

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Two lines of investigation supported the premise that killing of *Staphylococcus aureus*, 502A, by hydrogen peroxide involves formation of the more toxic hydroxyl radical (·OH) through the iron-dependent Fenton reaction. First, growing *S. aureus* overnight in broth media with increasing concentrations of iron increased their content of iron and dramatically enhanced their subsequent susceptibility to killing by H_2O_2. Second, in direct relation to their effectiveness as ·OH scavengers, thiourea, dimethyl thiourea, sodium benzoate, and dimethyl sulfoxide inhibited H_2O_2-mediated killing of *S. aureus*.

The crucial role of iron in the resolution between microbial invader and host remains unsettled (1). A majority of the evidence indicates that hypoferremia is mainly beneficial to the host. This conclusion is largely based on knowledge that iron is an essential growth requirement for microbial organisms but is also supported by observations suggesting an increased propensity to infection during hyperferremic episodes, such as those occurring during liver destruction, hemolytic anemia, and other disorders. Support for the possibility that iron helps the host relates primarily to findings that severe hypoferremia may impair host defense mechanisms, such as the killing of microorganisms by phagocytic cells (2).

To further investigate the possible biochemical role of iron in bacterial host defense interactions, we recently grew bacteria in iron before incubating them with neutrophils (3). We found that while preincubating *Staphylococcus aureus* in iron did not increase their susceptibility to killing by neutrophils, it markedly increased their susceptibility to killing by hydrogen peroxide (3).

In the present investigation we tried to elucidate the mechanism underlying this finding by addressing the hypothesis that iron in microorganisms facilitates bactericidal mechanisms by reacting with H_2O_2 to form the more toxic hydroxyl radical (·OH). Production of hydroxyl radical occurs by the well known Fenton reaction (4-7),

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\cdot}OH + OH^-$$

The results of the present investigation supported this premise. Increasing the concentration of iron in *S. aureus* increased their susceptibility to killing by H_2O_2. Scavengers of ·OH inhibited killing of *S. aureus* by H_2O_2 and in direct relation to their effectiveness as ·OH scavengers. This mechanism may be a new way in which iron is beneficial to the host.

EXPERIMENTAL PROCEDURES

S. aureus, 502A, were initially grown overnight (18 h) at 37 °C in Bacto nutrient broth media with increasing concentrations of $FeSO_4$, harvested, washed thoroughly, and their iron content (micrograms/10^9 organisms) determined using the ferozine method (8). Mean iron concentrations of the overnight broth media of 0.3, 3.3, or 6.5 μg/ml yielded *S. aureus* with mean iron concentrations of 0.2, 0.7, or 1.7 μg/10^9 organisms, respectively. Each iron value is the mean of 15 to 20 determinations. Killing was measured by mixing *S. aureus* (5×10^6) in human serum albumin (0.1%) and Hanks' balanced salt solution (1 ml) with or without H_2O_2 (9). After mixing, each tube was immediately sampled by micropipette, capped, and incubated at 37 °C. After 60 min, each tube was resampled. Samples were diluted and plated on agar. After 24 h, the number of colonies on each plate was counted electronically (Automatic Colony Counter, 3M, Minneapolis, MN). The percentage of the initial inoculum of *S. aureus* killed was calculated from 0- and 60-min plate counts and expressed as the mean ± S.E. Each LD_{50} point on the graph is the average ± S.E. of 12 determinations.

Methane production from Me_2SO[1] was measured by gas chromatography as previously described (10). In some samples, γ-irradiation was administered for 2 min at 0 °C in closed glass vials in a γ cell ^{60}Co source delivering a dose of roughly 1.8 krad/min (18 γ-rays/min) as described (11). CH_4 production from Me_2SO which has been irradiated is a relatively specific consequence of reaction of Me_2SO with ·OH (10, 11) and, therefore, is an acceptable way of assessing the relative ·OH scavenging abilities of these inhibitors under our test conditions.

RESULTS

Two lines of investigation supported the premise that killing of *S. aureus* by H_2O_2 involves production of the more toxic hydroxyl radical by an iron-dependent Fenton mechanism. First, preincubation of *S. aureus*, 502A, overnight (18 h) in broth media with increasing concentrations of $FeSO_4$ progressively increased their intrinsic content of iron and greatly enhanced their subsequent susceptibility to killing by H_2O_2 in the absence of extrinsic iron added during the bactericidal assay (Fig. 1). In contrast, addition of iron (0.3 to 6.5 μg/ml) during the bactericidal assay did not appreciably increase killing by H_2O_2 of *S. aureus* initially grown overnight in broth media without added iron (data not shown). Thus, with respect to susceptibility to H_2O_2, the intrinsic iron concentrations of the *S. aureus* are much more important than the amount of iron present extrinsically.

The suggested role of iron was further supported by control studies showing that direct alterations in the growth rates, viability, and catalase activities of *S. aureus* grown in increasing concentrations of iron did not account for their amplified sensitivity to killing by H_2O_2. Specifically, adding $FeSO_4$ to

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[1] The abbreviation used is: Me_2SO, dimethyl sulfoxide.

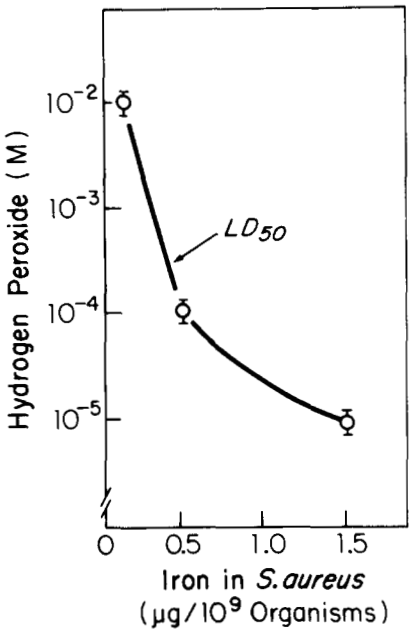

FIG. 1. **Hydrogen peroxide needed to kill 50% of the initial inoculum of *S. aureus* (LD_{50}) as a function of the intrinsic iron (micrograms/10^9 organisms) of the *S. aureus* (○——○).** Increasing the iron content of the *S. aureus* markedly increased their susceptibility to killing by H_2O_2. Each LD_{50} point on the graph is the mean ± S.E. of 12 determinations.

their overnight broth media did not change the growth rates or viability of *S. aureus* during the bactericidal assay. The numbers of *S. aureus* in 10-μl samples taken from *S. aureus* grown in broth mixtures with 0.3 or 6.5 μg/ml of iron were the same after 15 (2338 ± 42 *versus* 2267 ± 74), 17 (2194 ± 42 *versus* 2201 ± 29), 19 (2210 ± 125 *versus* 1925 ± 126), or 20.5 (1954 ± 67 *versus* 1875 ± 53) h of incubation (6 determinations for each value). In the absence of H_2O_2, the viable numbers of *S. aureus* at the end of the 1-h killing assay were 1.05 ± 0.09 times the numbers at the beginning of the bactericidal assay. This was true whether *S. aureus* had been previously grown overnight in media containing either 0.3 or 6.5 μg/ml of iron (12 determinations). In addition, preliminary growth in broth media with 0.3 or 6.5 μg/ml of iron did not change the catalase activities of *S. aureus* which were, respectively, 2.6 ± 0.2 or 2.8 ± 0.2 μM H_2O_2 decomposed/10^9 *S. aureus*/min (5 determinations) (12). Furthermore, since no peroxidase activity was found in either high iron or low iron *S. aureus*, it is unlikely that the observed alterations in killing involved Fe-mediated alterations in peroxidase (13).

The second line of evidence implicating participation of ·OH in killing of *S. aureus* by H_2O_2 was derived from experiments showing that ·OH scavengers (14) decreased killing of high iron *S. aureus* (1.7 μg/10^9 organisms) by H_2O_2 (Table I), as well as low iron *S. aureus* (0.2 μg/10^9 organisms, data not shown), in close relationship to their effectiveness as ·OH scavengers (Table II). Specifically, increasing concentrations of thiourea, dimethyl thiourea, sodium benzoate, and dimethyl sulfoxide decreased killing of high iron (1.7 μg/10^9 organisms) *S. aureus* by H_2O_2 (Table I). In complementary parallel studies, comparable 100 mM concentrations of thiourea, dimethyl thiourea, and sodium benzoate inhibited irradiation-generated ·OH-mediated CH_4 production from Me_2SO (11) while mannitol, urea, dimethyl urea, and superoxide dismutase had no inhibitory effect (Table II). Thus, the relative effectiveness of these scavengers in inhibiting H_2O_2-mediated killing of *S. aureus* by H_2O_2 was the same as their relative ability to inhibit

TABLE I

Effect of inhibitors on killing of S. aureus by hydrogen peroxide

Inhibitor added	Inhibition of bactericidal activity (% of control with no inhibitor added)
Thiourea (1.5 mM)	7 ± 3 (18)[a,b]
Thiourea (5.0 mM)	72 ± 5 (22)[b]
Thiourea (15 mM)	98 ± 1 (14)[b]
Thiourea (100 mM)	96 ± 2 (12)[b]
Dimethyl thiourea (2.5 mM)	19 ± 2 (14)[b]
Dimethyl thiourea (5.0 mM)	35 ± 2 (14)[b]
Dimethyl thiourea (10 mM)	61 ± 3 (14)[b]
Dimethyl thiourea (100 mM)	96 ± 2 (14)[b]
Sodium benzoate (100 mM)	26 ± 6 (12)[b]
Me_2SO (100 mM)	8 ± 3 (12)[c]
Me_2SO (140 mM)	19 ± 2 (11)[b]
Me_2SO (280 mM)	38 ± 3 (12)[b]
Mannitol (50 mM)	0 (12)[c]
Mannitol (100 mM)	6 ± 3 (12)[c]
Urea (15 mM)	0 (6)[c]
Urea (100 mM)	6 ± 1 (12)[c]
Dimethyl urea (100 mM)	0 (14)[c]
Superoxide dismutase (10 μg/ml)	2 ± 1 (16)[c]
Superoxide dismutase (1 μg/ml)	0 (18)[c]

[a] Mean ± S.E. (number of determinations) from tubes containing *S. aureus* (5 × 10^6), H_2O_2 (8.82 × 10^{-5} M), human serum albumin (0.1%), and the inhibitors. In the absence of inhibitors, H_2O_2 killed 52 ± 3.6% of the initial inoculum of *S. aureus* (30 determinations).

[b] Value significantly different ($p < 0.05$) from value with no inhibitor added.

[c] Value not significantly different ($p > 0.05$) from value with no inhibitor added.

TABLE II

Effect of inhibitors on production of CH_4 from Me_2SO exposed to irradiation

Inhibitor added	Inhibition of CH_4 production (% of control with no inhibitor added)
Thiourea (100 mM)	37 ± 5 (5)[a,b]
Dimethyl thiourea (100 mM)	31 ± 3 (5)[b]
Sodium benzoate (100 mM)	18 ± 2 (5)[b]
Me_2SO (100 mM)[c]	
Mannitol (100 mM)	0 (5)[d]
Urea (100 mM)	0 (5)[d]
Dimethyl urea (100 mM)	0 (5)[d]
Superoxide dismutase (100 μg)	0 (5)[d]

[a] Mean ± S.E. (number of determinations) from tubes containing Me_2SO (100 mM), human serum albumin (0.1%), and the inhibitor. In the absence of inhibitors, irradiation produced 0.32 ± 0.04 pmol of CH_4 (12 determinations).

[b] Value significantly different ($p < 0.05$) from value with no inhibitor added.

[c] DMSO was not tested against itself.

[d] Value not significantly different ($p > 0.05$) from value with no inhibitor added.

irradiation-generated ·OH-mediated CH_4 production from Me_2SO.

Additional control observations supported the relative specificity of the inhibitors. First, the active inhibitors did not decrease killing by directly altering viability of *S. aureus*. Specifically, in the absence of H_2O_2, the numbers of *S. aureus* present at the end of the bactericidal assay were the same with or without the inhibitors (data not shown). Second, under our test conditions, neither thiourea, dimethyl thiourea, sodium benzoate, nor Me_2SO reacted directly or significantly with H_2O_2 as monitored by optical density at 240 nm (12). In addition, methane, a product of the interaction of ·OH and Me_2SO (10, 11), was detectable in mixtures of *S. aureus* + H_2O_2 + Me_2SO or $FeSO_4$ + H_2O_2 + Me_2SO, but not detectable in mixtures of H_2O_2 + Me_2SO or $FeSO_4$ + Me_2SO or the above substances individually (data not shown) (10, 11).

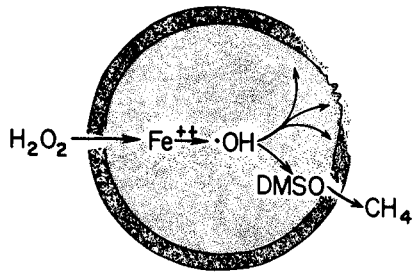

FIG. 2. **Schematic diagram of the proposed mechanism for killing of bacteria by hydrogen peroxide.** H_2O_2 reacts with bacterial iron (Fe^{2+}) to form the more toxic hydroxyl radical which damages the bacteria. Scavengers of ·OH, such as dimethyl sulfoxide (*DMSO*), react and inactivate ·OH and protect bacteria from killing by H_2O_2. When Me_2SO is the scavenger, methane, a product of the interaction of Me_2SO and ·OH, is produced.

DISCUSSION

Our results indicate that hydroxyl radical may contribute to killing of *S. aureus* by H_2O_2 (Fig. 2). Production of ·OH appeared to involve reaction of H_2O_2 with Fe^{2+} which had been previously incorporated by *S. aureus*. These statements are corroborated primarily by experiments showing that killing by H_2O_2 was clearly proportional to the intrinsic iron content of the *S. aureus* and that increasing concentrations of ·OH scavengers increasingly inhibited killing of *S. aureus* by H_2O_2. The additional observation that the degree of inhibition by these agents was proportional to their purported effectiveness as ·OH scavengers further supported this premise. Thus, while iron is an essential growth nutrient, it may also provide an "Achilles heel" for *S. aureus*. Thus, even though *S. aureus* have large amounts of catalase (15), reaction of unscavenged H_2O_2 with Fe^{2+} could produce ·OH at strategic locations in the bacterium (4–7). Since there are no known enzymatic scavengers of ·OH and presumed nonenzymatic scavengers may not be fully protective, even small amounts of the highly toxic ·OH produced by this mechanism might be very effective in killing microbial invaders (16–18). Furthermore, one might speculate that this proposed mechanism may contribute to the toxicity of H_2O_2 to other tissues, such as erythrocytes (19), tumor cells (20), or the lung (21).

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