Superoxide Dismutase Amplifies Organismal Sensitivity to Ionizing Radiation*

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Although increased superoxide dismutase (SOD) activity is often associated with enhanced resistance of cells and organisms to oxidant challenges, few direct tests of the antioxidative importance of this enzyme have been carried out. To assess the importance of SOD in defending against γ-radiation, we employed Escherichia coli with deficient, normal, and super-normal enzyme activities. Surprisingly, the radiation sensitivity of E. coli actually increases as bacterial SOD activity increases. Elevated intracellular SOD activity sensitizes E. coli to radiation-induced mortality, whereas SOD-deficient bacteria show normal or decreased radiosensitivity. Toxic effects of activated oxygen species are involved in this phenomenon; bacterial SOD activity has no effect on radiation sensitivity under anaerobic conditions or on the lethality of other, non-oxygen-dependent, toxins such as ultraviolet radiation.

In biological materials, ionizing radiation causes the generation of reactive oxygen species. Indeed, radiation generates the same species of activated oxygen which occur spontaneously in the course of aerobic life (1); these include superoxide (O2·−), hydrogen peroxide (H2O2), and hydroxyl radical (·OH). The oxidant defense enzymes (superoxide dismutase (SOD),1 catalase, and glutathione peroxidase) may therefore be of importance in determining the resistance of aerobes to ionizing radiation. The importance of reactive oxygen species in radiation damage is emphasized by the enhancement of radiation toxicity (2-3-fold) under aerobic versus anaerobic conditions (2). In the presence of oxygen, O2·− is readily formed by ionizing radiation, suggesting that O2·− or a derivative thereof may participate in the oxygen-dependent enhancement of radiation toxicity. Consequently, it has been proposed that enzymes which catabolize these species, especially SOD, may be important in resistance to ionizing radiation (3-10).

SOD, which converts O2·− to H2O2, might function as a radioprotectant by removing O2·−, thereby either neutralizing a potentially dangerous radical or preventing the redox cycling of metals involved in the generation of ·OH via the Haber-Weiss reaction. In support of this proposition, previous studies (9-11) have demonstrated that additions of extracellular SOD and/or catalase partially protect both prokaryotic and eukaryotic organisms against oxygen-mediated radiation damage. However, the importance of intracellular SOD as a radioprotectant has not been fully assessed. To this end, we exposed Escherichia coli having deficient, normal, and very high SOD activity to ionizing radiation. Surprisingly, our results suggest that SOD actually increases the lethality of radiation under aerobic, but not anaerobic, conditions.

MATERIALS AND METHODS

E. coli express an iron (FeSOD) and a manganese (MnSOD) SOD (12, 13). Both SODs are cytoplasmic (14) and have similar second-order rate constants of catalysis (15). To assess the importance of intracellular SOD in moderating the O2·−-dependent toxicity of γ-radiation, normal, FeSOD-rich E. coli strain LE392 (16, 17) and FeSOD-, MnSOD-, and Fe/MnSOD-deficient clones of E. coli strain GC4468 (18) were irradiated, and the extent of radiation-dependent bacterial killing was assessed.

Three clones of E. coli strain LE392 were employed: normal SOD (parental strain), high SOD (transformed with multiple copies of the pHS1.4 plasmid containing the gene for bacterial FeSOD) (16), and a plasmid control (transformed with multiple copies of pBR322 plasmid DNA) clones with normal SOD activity. In addition, four clones of E. coli GC4468 were employed. These clones consisted of the parental (normal SOD), sodA− (MnSOD-deficient), sodB− (FeSOD-deficient), and sodA−B− (MnSOD- and FeSOD-deficient) bacteria (18). Both E. coli strains are recA−. Selective pressure on the high SOD bacteria was maintained by tetracycline (12.5 μg/ml), whereas the plasmid control clone was maintained by tetracycline (12.5 μg/ml) and ampicillin (40 μg/ml). Selective pressure on the sodA−, sodB−, and sodA−B− clones was maintained by chloramphenicol (20 μg/ml), kanamycin (40 μg/ml), or both, respectively.

Overnight cultures of the bacteria were grown in brain heart infusion medium (BHI; Difco), inoculated with the appropriate antibiotic, at 37 °C in a shaking water bath. Test cultures of BHI medium (using 1 x 1010 E. coli/ml) were treated with 17 μg/ml chloramphenicol (LE392) or 0.5 mg/ml puromycin (GC4468) to prevent bacterial replication and de novo induction of protein synthesis which might otherwise occur during even brief exposure to radiation. Aerobic radiation sensitivity was determined by placing 10 ml of the test culture in a 10-cm Petri dish and irradiating in a Mark 1 137Cs irradiator (J. L. Sheppard and Associates, Glendale, CA) at a rate of 1 krad/min. Anaerobic cultures were prepared by overnight growth in vacuum-degassed (15 min) and nitrogen-sparged (15 min) BHI in sealed screw-top tubes. Radiation exposure was carried out in a similarly prepared medium in screw-top tubes inoculated with 1 x 107 bacteria/ml. Sample aliquots of aerobic and anaerobic cultures were removed, diluted, and plated on nutrient agar (Difco). Culture plates were inoculated at 37 °C for 24-36 h, and viable bacterial colony-forming units were counted on an Artek Systems Autocount bacterial plate counter. Lethal dose values were determined by linear regression, and statistical significance was determined by analysis of variance.

Enzymatic Analyses—Overnight cultures of bacterial clones were

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1The abbreviations used are: SOD, superoxide dismutase; FeSOD, iron-containing SOD; MnSOD, manganese-containing SOD; BHIM, brain heart infusion medium.
grown as previously described, concentrated by centrifugation (3000
× g), washed three times with 50 mM potassium phosphate buffer,
PH 7.0, resuspended to 1 × 10^8/ml, and sonicated at 40–50 watts for
90 s in a Branson sonicator. The crude sonicates were placed on ice
for immediate assay or frozen at −70 °C for later enzymatic analysis.
Enzyme activity is expressed as units of activity/milligram of protein.
Protein concentrations were determined by the method of Lowry et
al. (19).

For measurements of SOD activity, sonicated bacteria were centri-
fuged at 3000 × g. The supernatant was assayed for SOD activity
with the cytochrome c reduction method (20). Discrimination of
MnSod and FeSOD was done electrophoretically (21) and in direct
assays using the preferential inhibition of FeSOD by azide (22). Azide
(10 mM) inhibits >80% of the FeSOD activity while decreasing
MnSod activity by less than 15%.

Catalase activity was determined in bacterial sonicates by a mod-
ification of the method of Eaton et al. (22). Briefly, this assay detects
the catalase-mediated disappearance of H_2O_2 monitored spectropho-
tometrically at 240 nm. Freezing was found to diminish catalase
activity; for this reason, only fresh bacterial sonicates were used for
catalase determination. Catalase activity is expressed as units/milli-
gram of protein. Total glutathione content was measured on depro-
teinized and neutralized 10% trichloroacetic acid extracts of bacteria
using a sensitive enzymatic technique (24).

Iron Determination—Bacterial iron content was determined by the
Ferrozine method (25). Briefly, aliquots of bacterial sonicates were
added to an acidic iron reagent buffer which contained sodium dodecyl
sulfate to solubilize and denature proteins. The initial absorption at
562 nm was determined, after which Ferrozine (Hach Chemical Co.,
Ames, IA) was added to a final concentration of 138 μM. The samples
were incubated at 37 °C for 15 min, and ODmax was determined.

RESULTS

Values for various oxidant defense parameters of the ex-
perimental E. coli strains are shown in Table I. As shown, the
high SOD bacteria exhibit a >10-fold increase in SOD activity,
but have normal catalase activity. Similarly, no differ-
cences in catalase activity were noted among the SOD-deficient
E. coli. Finally, no significant differences were found in GSH
content of any of the strains employed, with values for GSH
varying from 15 to 20 μmol/mg of bacterial protein.

Surprisingly, the high SOD E. coli exhibit substantially
increased radiation sensitivity (Fig. 1A). However, when irra-
diation is carried out under anaerobic conditions, the parental,
plasmid (pBR control), and high SOD E. coli are equally
radioresistant (Fig. 1B). Therefore, the enhanced radiation
sensitivity of high SOD bacteria is very likely due to reactions
involving molecular oxygen.

Even more surprisingly, SOD-deficient E. coli are not more
radioresistant (Fig. 2). In fact, both the double mutant
(MnSOD- and FeSOD-deficient, sodA⁻B⁻) and the MnSOD-
deficient mutant (sodA⁻) proved to be significantly more
radioresistant than the parental strain. It should be noted that the enhanced radioresistance of the MnSOD-deficient

![Table I](https://example.com/table.png)

**TABLE I**

Antioxidant enzymes of experimental E. coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>Total SOD</th>
<th>FeSOD</th>
<th>MnSOD</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE392</td>
<td>10</td>
<td>11.9</td>
<td>10.6</td>
<td>1.3</td>
<td>15.4</td>
</tr>
<tr>
<td>Parental</td>
<td>10</td>
<td>12.6</td>
<td>11.1</td>
<td>1.5</td>
<td>15.8</td>
</tr>
<tr>
<td>pBR control</td>
<td>10</td>
<td>15.6^a</td>
<td>13.7</td>
<td>0.9</td>
<td>18.7</td>
</tr>
<tr>
<td>GC4468</td>
<td>10</td>
<td>13.2</td>
<td>12.1</td>
<td>1.1</td>
<td>9.2</td>
</tr>
<tr>
<td>sodA⁻</td>
<td>10</td>
<td>14.1</td>
<td>14.1</td>
<td>0.0</td>
<td>8.2</td>
</tr>
<tr>
<td>sodB⁻</td>
<td>4</td>
<td>1.5^b</td>
<td>0.0</td>
<td>1.5</td>
<td>7.9</td>
</tr>
<tr>
<td>sodA⁻B⁻</td>
<td>4</td>
<td>≤0.9^a</td>
<td>0.0</td>
<td>0.0</td>
<td>8.8</td>
</tr>
</tbody>
</table>

^a Significantly different (p < 0.001) from parental and pBR control

^b Significantly different (p < 0.001) from parental and sodA⁻
(MnSOD-deficient) clones.

As would be expected, high FeSOD bacteria have increased
intracellular iron, and we suspected that this might be par-
tially responsible for the enhanced aerobic radiation sensiti-
vity. To evaluate this possibility, the bacterial iron concen-
tration was increased in all three clones of LE392 by growth in
2 mM ferric nitrilotriacetate. Growth under these conditions
resulted in increased iron concentrations of 25.7-, 53.4-, and
10.1-fold in the parental, plasmid control, and high SOD
bacteria, respectively (Table II). Radiation sensitivity of these
bacteria was determined using washed bacteria resuspended
in non-iron-supplemented BHIM. As shown in Table III, iron-
rich LE392 were actually more radioresistant. Similarly, the
radio sensitivities of the parental and SOD-deficient E. coli
GC4468 are unaffected (Table III). In aggregate, these results

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Effect of γ-irradiation on survival of aerobic (A) and anaerobic (B) cultures of parental (Δ), plasmid control (C), and high SOD (D) E. coli LE392. Chloramphenicol-treated bacterial cultures (1 × 10^8/ml) were irradiated in 10-cm Petri plates, at 1 krad/min, in a cesium irradiator. No significant difference was found between the parental and plasmid control E. coli; however, the high SOD bacteria showed a greater than 2-fold enhancement in radiosensitivity at all doses employed (p < 0.005). No significant difference was found between any of the clones upon anaerobic irradiation (B). Values shown represent the mean ± 1 S.D. of eight aerobic and five anaerobic experiments. CFU, colony-forming units.
plates, at parental strain or FeSOD-deficient clone at the highest radiation treated bacterial cultures. Values shown represent the mean ultraviolet radiation between the (FeSOD-deficient) Unsupplemented and MnSOD-deficient clones demonstrate significantly (p < 0.01 and < 0.05, respectively) enhanced radioresistance in comparison to the parental strain or FeSOD-deficient clone at the highest radiation dose employed. Values shown represent the mean ± 1 S.D. of four aerobic and three anaerobic experiments.

TABLE II
Iron levels of E. coli LE392

<table>
<thead>
<tr>
<th>n</th>
<th>Iron/protein µg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsupplemented BHIM</td>
<td>3 ± 0.192 ± 0.013</td>
</tr>
<tr>
<td>Iron-supplemented BHIM*</td>
<td>4.94 ± 0.27</td>
</tr>
</tbody>
</table>

*2 mM ferric nitriloacetate added.

As indicated, the increased radiosensitivity of the high SOD LE392 is not due to iron borne by this metalloenzyme, nor are the high SOD bacteria more sensitive to other toxicants. As shown in Fig. 3, there is no differential sensitivity to ultraviolet radiation between the LE392 clones.

DISCUSSION

Our results are, at least superficially, at variance with earlier observations in which SOD was reported to prevent radiation-mediated killing in both prokaryotes and eukaryotes (2, 6, 7, 9, 10, 26–28, 30, 32, 33). However, the importance of intracellular SOD as a radioprotectant is by no means certain; whereas several radioresistant strains of bacteria contain exceptionally high levels of SOD (34), it is not clear whether SOD per se is responsible for the enhanced radiation resistance. In fact, earlier studies (2, 36) indicate that induction of intracellular SOD, by prior oxidant challenge, does not appear to have any protective effect against ionizing radiation. On the other hand, treatment of Chinese hamster ovary cells with diethyl dithiocarbamate (DDC), which inactivates CuZnSOD, also results in increased radioresistance (4, 5). Whereas this suggests that CuZnSOD may be important in tempering radiation sensitivity, it is important to note that diethyl dithiocarbamate also inhibits glutathione peroxidase and depletes cellular reduced glutathione (37), an important radioprotector (29, 31, 38).

In fact, protection of cells or organisms against ionizing radiation by elevated intracellular SOD has not been directly demonstrated in any experimental system. Induction of intracellular SOD, by prior oxidant challenge, does not appear to have any significant protective effect against radiation in a number of bacterial models. It is, however, surprising that

<table>
<thead>
<tr>
<th>n</th>
<th>LE392 LD50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parental</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>BHIM</td>
<td>6</td>
</tr>
<tr>
<td>BHIM + chelator*</td>
<td>2</td>
</tr>
<tr>
<td>BHIM + Fe*</td>
<td>2</td>
</tr>
<tr>
<td>BHIM + Fe* + formate*</td>
<td>2</td>
</tr>
</tbody>
</table>

*B 5 mM o-phenanthroline.

*2 mM FeSO4 in 2 mM nitritotriacetic acid (used to facilitate iron uptake by the bacteria).

*10 mM formate.

*ND, not determined.

\[ \text{Percent Initial CFU} \]

\[ \text{Krads} \]

\[ \text{Minutes} \]

\[ \text{Parental} \]

\[ \text{Plasmid control} \]

\[ \text{High SOD} \]

\[ \text{Table III} \]

Effect of iron on radiation sensitivity

\[ \text{LE392 LD50} \]

\[ \text{GC4468 LD50} \]

\[ \text{n} \]

<table>
<thead>
<tr>
<th></th>
<th>Parental</th>
<th>sodA</th>
<th>sodB</th>
<th>sodA-B*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHIM</td>
<td>6</td>
<td>14.6</td>
<td>13.4</td>
<td>5.6</td>
</tr>
<tr>
<td>BHIM + chelator*</td>
<td>2</td>
<td>22.7</td>
<td>21.1</td>
<td>8.1</td>
</tr>
<tr>
<td>BHIM + Fe*</td>
<td>2</td>
<td>20.6</td>
<td>20.9</td>
<td>14.3</td>
</tr>
<tr>
<td>BHIM + Fe* + formate*</td>
<td>2</td>
<td>20.2</td>
<td>ND*</td>
<td>14.5</td>
</tr>
</tbody>
</table>

\[ *5 \text{ mM} \text{ o-phenanthroline.} \]

\[ *2 \text{ mM} \text{ FeSO}_4 \text{ in } 2 \text{ mM} \text{ nitritotriacetic acid (used to facilitate iron uptake by the bacteria).} \]

\[ *10 \text{ mM} \text{ formate.} \]

\[ *\text{ND, not determined.} \]
elevated SOD activity may actually enhance organismal radiosensitivity. Two secondary effects of rapid O$_2$ dismutation include increased susceptibility to O$_2$-generating drugs (paraquat) and hypoxia. Similar results have also been observed in $E$. coli with genetically elevated MnSOD activity. Second, the immediate products of aerobic radiation include O$_2^\cdot$ (a reductant) and \textsuperscript{1}OH, which may readily react, forming innocuous products (OH$^-$ and O$_2$). Rapid depletion of O$_2^\cdot$ by SOD may therefore enhance the net oxidative effects of radiation through consumption of reducing equivalents.

We should note that the increased radiation sensitivity of the high SOD $E$. coli is not attributable to elevated bacterial iron content. We tested this possibility because high FeSOD $E$. coli, grown in unsupplemented BHIM, contain roughly twice the iron of the parental and plasmid control $E$. coli. However, normal SOD $E$. coli with 40 times the normal iron content are not more sensitive to radiation, and elevated iron concentration in the high SOD $E$. coli actually decreases the radiosensitivity of these organisms. Finally, $E$. coli with exaggerated FeSOD activity are not simply more sensitive to any toxic challenge; no differences were found in the rate of killing of our experimental $E$. coli by short-wave UV irradiation.

This study supports the idea that enhanced SOD activity alone does not prevent oxidant damage caused by ionizing radiation. In fact, exaggerated SOD activity accelerates oxygen-mediated radiation damage. Similarly, deficiency of either FeSOD or MnSOD does not enhance radiation damage. Indeed, MnSOD-deficient mutants actually appear to be radioresistant. These unexpected observations may be explained by one of two potentially adverse effects of high SOD activity. First, SOD-driven accumulation of H$_2$O$_2$ may explain the preferential toxicity of \gamma-irradiation to high SOD $E$. coli as well as the finding that SOD-deficient $E$. coli are not more susceptible to ionizing radiation. Second, elevated SOD may deplete a reducing species, O$_2^\cdot$, which would otherwise react with \textsuperscript{1}OH, which is also generated in large amounts of \gamma-radiation. In either case, SOD, a putative \textsuperscript{"an"}tioxidant enzyme, may function to actually enhance radiation-induced oxidant damage. However, our observations should not be taken to indicate that SOD has no important function in organismal oxidant defense. Rather, our present and earlier (17) investigations suggest that the metabolism of activated oxygen must be viewed as a biochemical pathway in which the balance of oxidant defense functions is more important than adjustments of individual parts of the system. This may be particularly true in the case of SOD, which produces a potentially toxic product (H$_2$O$_2$), and may explain why SOD is closely regulated in aerobic organisms. These results may necessitate a re-evaluation of the advisability of supplementing SOD activity therapeutically to effect improvements in cellular or organismal oxidant defense.

Acknowledgments—We thank Drs. Danièle Touati and Joe McCord for helpful discussions.

REFERENCES