NOTES

Hyperoxia and Prolongation of Aminoglycoside-Induced Postantibiotic Effect in *Pseudomonas aeruginosa*: Role of Reactive Oxygen Species

MATTHEW K. PARK, ROY A. M. MYERS, AND LOUIS MARZELLA*

Department of Pathology, School of Medicine, University of Maryland, and Maryland Institute for Emergency Medical Services Systems, 10 South Pine St., Baltimore, Maryland 21201

Received 20 July 1992/Accepted 30 September 1992

Hyperoxia prolongs the postantibiotic effect (PAE) of the aminoglycoside tobramycin in *Pseudomonas aeruginosa*. We tested the hypothesis that the PAE is prolonged because hyperoxia increases free radical flux while tobramycin inhibits the induction of antioxidant defenses. Exposure of *P. aeruginosa* to hyperoxia (100% O2) for 1 h increased superoxide dismutase, catalase, and glutathione levels. In the presence of tobramycin (1 × the MIC), the induction of antioxidant defenses by hyperoxia was nearly abrogated. Neither preexposure of *P. aeruginosa* to hyperoxia nor supplementation with the antioxidants copper(II) (diisopropylsalicylate)2 (superoxide dismutase-like), catalase, or dimethyl sulfoxide abolished prolongation of the PAE of tobramycin induced by hyperoxia.

We recently showed that hyperoxia acts in synergy with an aminoglycoside antibiotic to prolong the postantibiotic effect (PAE) of tobramycin against *Pseudomonas aeruginosa* (18). The PAE describes the period of bacterial growth suppression that results from a brief exposure to an antimicrobial agent (14). For aminoglycosides, the PAE represents the time required for synthesis of new ribosomes (4).

Hyperoxia increases the intracellular flux of O2− and other reactive oxygen species (8). In the study described here, we tested the hypothesis that hyperoxia enhances the PAE of tobramycin against *P. aeruginosa* by increasing the intracellular flux of reactive oxygen species at a time when synthesis of new antioxidant enzymes is inhibited. To this end, superoxide dismutase (SOD) and catalase (CAT) activities and glutathione (GSH) levels were measured after exposure to hyperoxia and/or tobramycin (1 × the MIC) to determine whether the stress caused by hyperoxia induces an adaptive response in *P. aeruginosa*. In order to assess the role of specific reactive oxygen species in the enhancement of the PAE of tobramycin by hyperoxia, the following antioxidants were added to the bacterial culture: copper(II) (diisopropylsalicylate)2 (CuDIPS; a lipophilic SOD-mimicking agent), CAT (which reduces H2O2 to O2 and H2O), and dimethyl sulfoxide (DMSO; a lipophilic scavenger of hydroxyl radicals).

*P. aeruginosa* ATCC 27853 was obtained from the American Type Culture Collection (Rockville, Md.). Logarithmic-phase bacteria were optically matched with a 0.5 McFarland standard and were diluted to a final concentration of 107 CFU/ml in prewarmed, cation-supplemented Mueller-Hinton broth (17). The following four assay conditions were examined: 21% O2, 21% O2 plus tobramycin, hyperoxia (100% O2, 101.3 kPa), and hyperoxia plus tobramycin. The MIC of tobramycin for *P. aeruginosa* ATCC 27853 was 0.5 μg/ml.

The antioxidant enzymes and free radical scavengers were used at the highest concentration which did not inhibit bacterial growth, namely, CuDIPS at 1 × 10−4 M, CAT at 4 × 10−7 M, and DMSO at 0.4 M. The antioxidants were added before exposure to hyperoxia. The PAE was measured as described previously (18).

For enzymatic and thiol assays, bacteria (final concentration, ~107 CFU/ml) were grown for 2 h in a shaking water bath under ambient conditions (21% O2, 37°C, 100 rpm) before a 1-h exposure to hyperoxia and/or tobramycin (1 × the MIC). Bacteria were then filtered onto 0.2-μm-pore-size filters, washed, and resuspended in 50 mM phosphate buffer (pH 7.0); filters were removed after vortexing. Bacteria were centrifugated at 10,000 × g for 15 min at 4°C. After decanting and resuspending with 50 mM phosphate buffer (pH 7.0) with 0.1 mM EDTA, bacterial cells were lysed by sonication (model W185 sonifier; Branson, Plainview, N.Y.) at 60 W (45 s, eight times). Membranes were sedimented by centrifugation at 27,500 × g for 30 min.

SOD was assayed spectrophotometrically by measuring the rate of cytochrome c reduction by O2− (generated by xanthine and xanthine oxidase) (7). CAT activity was quantified spectrophotometrically by measuring the decrease in the absorbance of H2O2 (1). Proteins were quantified using the bicinchoninic acid reagent (Pierce, Rockford, Ill.). Total soluble thiols (a measure of GSH) were measured spectrophotometrically at 535 nm (19). The Kruskal-Wallis analysis of variance by ranks and the multiple comparisons test were used for statistical analysis.

Table 1 shows that the activity of SOD (23.5 U/mg) in *P. aeruginosa* grown under normoxic conditions is comparable to that found in *Escherichia coli* K-12 (11); CAT activity (19.2 U/mg) was slightly greater. A brief, 1-h exposure to hyperoxia increased SOD (1.4-fold; P < 0.001) and CAT (2-fold; P < 0.02) activities in *P. aeruginosa*. Tobramycin

---

* Corresponding author.
abolished the induction of both SOD and CAT activities by hyperoxia. Interestingly, SOD activity in normoxia-exposed, tobramycin-treated bacteria increased 1.2-fold ($P < 0.05$) more than that in normoxia-exposed controls. Table 1 also shows that the levels of total soluble thiols, a measure of GSH content, in *P. aeruginosa* (1.4 nmol/mg) were approximately twice those reported in *E. coli* (16). Hyperoxia increased the level of total soluble thiols (6.4-fold; $P < 0.001$) in *P. aeruginosa* compared with those in normoxia-exposed controls. However, this 6.4-fold increase in thiols was inhibited by 50% ($P < 0.05$) when tobramycin was present in the growth medium. Tobramycin also increased thiol levels (4.8-fold; $P < 0.001$) in normoxia-exposed bacteria.

We exposed bacteria to hyperoxia (for 1 or 18 h) before examining the PAE to determine whether increased antioxidan levels could prevent the hyperoxic enhancement of the tobramycin-induced PAE. Table 2 shows that in the preexposed group, the PAE under hyperoxic conditions remained prolonged ($P < 0.001$) compared with that under normoxic conditions. Table 2 also shows that the addition of antioxidants (CuDIPS, CAT, and DMSO) did not diminish the hyperoxic enhancement of the PAE.

In bacteria, reactive oxygen species generated under hyperoxic conditions induce antioxidant defenses that are under the positive control of the soxR and oxyR regulons (12, 21). Approximately 30 proteins are induced by the soxR regulon in response to O$_2$ (22). Forty proteins are induced by the oxyR regulon in response to H$_2$O$_2$ (3, 9). Hyperoxia rapidly increased the activities of SOD and CAT in *P. aeruginosa*. This finding suggests that hyperoxia may have induced both the soxR and oxyR regulons in *P. aeruginosa*. Increased synthesis of these antioxidant enzymes by hyperoxia did not occur in the presence of the aminoglycoside tobramycin.

The induction of antioxidant enzymes by hyperoxia in the obligate aerobe that we examined (*P. aeruginosa*) appears to be similar to the induction described for facultative anaerobes (10, 11). The influence of hyperoxia on another critical antioxidant defense system, namely, the cellular GSH pool, to our knowledge has not been studied. In the presence of hyperoxia, we expected to find a decrease in the levels of GSH, because reactive oxygen species are known to deplete the GSH pool (20). Surprisingly, we found that hyperoxia increased GSH levels sixfold in *P. aeruginosa*. We postulate that platelet during exposure to hyperoxia, the increased flux of free radicals induces the enzymes γ-glutamylcysteine synthetase and GSH synthetase, which are responsible for the synthesis of GSH. Therefore, in addition to inducing GSH reductase, which is involved in maintaining the reduced pool of glutathione (15), the oxyR regulon may also induce enzymes involved in GSH biosynthesis. Support for this notion awaits the characterization of the many unknown proteins under control of the oxyR regulon. Tobramycin inhibited the hyperoxia-induced increase in GSH levels by 50%, supporting a role for increased enzyme biosynthesis in the increase in the intracellular pool of GSH. The increase in GSH levels in *P. aeruginosa* in response to the sublethal stress of hyperoxia is in agreement with several reports that in bacteria, GSH protects against killing by the free radicals generated by irradiation (16).

Interestingly, GSH levels in *P. aeruginosa* were also significantly increased by tobramycin under normoxic and hyperoxic conditions. GSH levels were 10-fold higher in *E. coli* grown to the early stationary growth phase compared with those in *E. coli* in the early logarithmic phase of growth (6, 16). Under these conditions, the mechanism for the increase in the size of the GSH pool is an increase in the intracellular level of cysteine available for GSH synthesis (13). The addition of cysteine to bacteria in the logarithmic phase of growth also induced an increase in GSH levels (13). These findings support the notion that in *P. aeruginosa*, tobramycin increases GSH levels by inhibiting logarithmic-phase growth.

The deleterious effects of oxygen-based free radicals on proteins are well characterized. In *E. coli*, denatured proteins are more susceptible to recognition and degradation by ATP-independent, nonlysosomal enzymes (5). Certain critical proteins may be more susceptible to oxidative damage or modification (2, 20). In *E. coli*, hydrogen peroxide oxidized all three methionine residues of ribosomal protein L12 to methionine sulfoxide, thereby converting the active dimer.

### Table 1. Effects of hyperoxia and tobramycin on SOD and CAT activities and total soluble thiols in *P. aeruginosa* ATCC 27853

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Antioxidant enzyme activity (U/mg)$^*$</th>
<th>Soluble thiol concn (nmol/mg)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SOD</td>
<td>CAT</td>
</tr>
<tr>
<td>I</td>
<td>21% O$_2$</td>
<td>23.5 ± 1.6</td>
<td>19.2 ± 3.9</td>
</tr>
<tr>
<td>II</td>
<td>21% O$_2$ + tobramycin</td>
<td>28.5 ± 1.3</td>
<td>16.1 ± 1.0</td>
</tr>
<tr>
<td>III</td>
<td>100% O$_2$</td>
<td>33.4 ± 2.1</td>
<td>38.3 ± 4.4</td>
</tr>
<tr>
<td>IV</td>
<td>100% O$_2$ + tobramycin</td>
<td>25.6 ± 1.2</td>
<td>21.6 ± 1.9</td>
</tr>
</tbody>
</table>

$^*$ Numbers represent means ± standard errors of the means of three to six determinations. Letters denote significant differences. The $P$ values and the comparison groups within each column are as follows:

- $^a P < 0.05$, group I.
- $^b P < 0.001$, group I.
- $^c P < 0.02$, group I.
- $^d P < 0.01$, group II.
- $^e P < 0.01$, group III.
- $^f P < 0.05$, group III.
- $^g P < 0.05$, group I.
- $^h P < 0.05$, group I.
- $^i P < 0.05$, group III.

### Table 2. Effects of hyperoxia preexposure and antioxidants on the PAE of tobramycin in *P. aeruginosa*

<table>
<thead>
<tr>
<th>Condition (no. of exp.)</th>
<th>PAE (h)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21% O$_2$$^b$</td>
</tr>
<tr>
<td>No preexposure (9)$^d$</td>
<td>0.70 ± 0.13</td>
</tr>
<tr>
<td>Hyperoxia preexposure (7)$^f$</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td>Antioxidants CuDIPS (3)</td>
<td>0.24 ± 0.20</td>
</tr>
<tr>
<td>CAT (3)</td>
<td>0.56 ± 0.17</td>
</tr>
<tr>
<td>DMSO (3)</td>
<td>0.47 ± 0.11</td>
</tr>
</tbody>
</table>

$^a$ Values are means ± standard errors of the mean and represent the duration of the PAE.

- $^b$ Bacteria were exposed to 21% O$_2$ and tobramycin (4× the MIC) for 1 h and were maintained in 21% O$_2$ after the removal of tobramycin.
- $^c$ Significantly different from data for bacteria under normoxic conditions ($P < 0.01$).
- $^d$ Bacteria were preexposed to hyperoxia before the PAE was determined.
- $^e$ Significantly different from data for bacteria under normoxic conditions ($P < 0.01$).
- $^f$ Significantly different from data for bacteria under normoxic conditions ($P < 0.02$).
- $^g$ Data are from Park et al. (18).
- $^h$ Antioxidants were exposed to 100% O$_2$ (101.3 kPa) and tobramycin (4× the MIC) for 1 h and were returned to normoxic conditions (21% O$_2$) after the removal of tobramycin.
- $^i$ Antioxidants were preexposed to hyperoxia before the PAE was determined.
form to the inactive monomer form (2). Binding of oxidized L12 to the 50S ribosomal subunit inhibited in vitro translation of polyphenylalanine. This oxidative modification is reversible (2). In the presence of protein synthesis inhibitors, oxidative damage to proteins with high turnover rates is likely to have more rapid and long-lasting effects on cell function.

These considerations suggest that the time required for repair or resynthesis of oxidatively modified cellular proteins may account for the enhanced PAE by hyperoxia of antimicrobial agents that are protein synthesis inhibitors. However, CuDIPS, CAT, and DMSO did not block the prolongation of the PAE of tobramycin by hyperoxia. In addition, preexposure of bacteria to hyperoxia before determining the PAE increased the intracellular SOD and CAT activities and GSH levels. Yet, the enhancement of the PAE was not affected by this modulation of antioxidant defenses.

We conclude that a short exposure to hyperoxia induces antioxidant defenses in P. aeruginosa. However, there is no evidence that hyperoxia acts synergistically with protein synthesis inhibitors through free radical mechanisms to inhibit bacterial growth after a short antibiotic exposure. It appears more likely that the synergistic effects of hyperoxia with antimicrobial agents that are protein synthesis inhibitors (18) are mediated by an inhibitory effect of hyperoxia on protein synthesis.

We thank T. W. Kensler (The Johns Hopkins University, Baltimore, Md.) for providing CuDIPS.

REFERENCES