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Superoxide protects *Escherichia coli* from bleomycin mediated lethality

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Abstract

Superoxide and its products, especially hydroxyl radical, were recently proposed to be instrumental in cell death following treatment with a wide range of antimicrobials. Surprisingly, bleomycin lethality to *Escherichia coli* was ameliorated by a genetic deficiency of superoxide dismutase or by furnishing the superoxide generator plumbagin. Rescue by plumbagin was similar in strains containing or lacking *recA* or with inactive, inducible, or constitutive *soxRS* regulons. Thus, superoxide interferes with bleomycin cytotoxicity in ways not readily explained by genetic pathways expected to protect from oxidative damage.

Keywords

Antibiotic; Iron; Oxygen; ROS; oxidative stress

1. Introduction

While lethal antibacterial agents are generally thought to act by damaging specific target biomolecules, the possibility has been raised that they may also indirectly elicit lethal oxidative damage [1–3]. For example, treatment with lethal antimicrobials may cause an inappropriate stress response, such as a burst of aerobic respiration [1] that generates superoxide radical ($\cdot\text{O}_2^-$) as a byproduct [4]. Such a response and the resulting increase in reactive oxygen species depend on the availability of O_2 for reduction by one electron to superoxide, providing a rationale for several very different antibacterials being more lethal under aerobic than anaerobic conditions [2,5]. Bleomycin (a mutagen and *in vivo* DNA-cleaving agent) may represent a special case of oxygen participation, since bleomycin utilizes O_2 for activation and also requires it for oxidative DNA cleavage. Furthermore, both superoxide and peroxide function as potential cofactors of bleomycin activation *in vitro* (Scheme) and may do so *in vivo*. Thus, O_2 and its reactive products may contribute at several levels to bleomycin cytotoxicity.

Superoxide is expected to stimulate lethality in two ways. First, increased superoxide can increase peroxide concentration through its spontaneous or enzymatic dismutation ($2\cdot\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$) [7], thereby fostering production of highly toxic hydroxyl radical, a

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peroxide reduction product. Second, superoxide can participate directly in the formation of “activated bleomycin” (Scheme), the drug intermediate that is kinetically competent for DNA oxidation *in vitro* [6,8]. Thus, superoxide enhances the ability of bleomycin to cleave purified DNA *in vitro*, whether supplied as the superoxide salt KO₂ [9] or generated enzymatically. Conversely, addition of superoxide dismutase (SOD) blocks bleomycin action that is otherwise facilitated by concentrated preparations of microsomes or nuclei [10–15]. Presumably, added SOD prevents superoxide-mediated microsomal formation of activated bleomycin. Superoxide might thereby have a variety of stimulating effects on bleomycin cytotoxicity.

In the present work we perturbed intracellular superoxide concentration in two ways to assess its effect on bleomycin lethality. In a genetic approach, *Escherichia coli* strains having different levels of superoxide dismutase were compared for susceptibility to bleomycin. In a second, metabolic approach bacterial cultures were compared for lethal bleomycin susceptibility during treatment with plumbagin, a natural naphthoquinone that is taken up and metabolized with the formation of superoxide [16]. Using either approach we were surprised to find that bleomycin toxicity was antagonized whenever treatment was expected to raise superoxide levels. These data suggest that superoxide can play a protective role in the response of bacteria to lethal stress, at least from bleomycin.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

E. coli strains and plasmids used in the study are listed in Table 1. New, plasmid-containing strains were constructed by bacterial transformation and by P1-mediated transduction. Bacteria were grown aerobically in LB liquid medium and on LB agar [17] at 37 °C. In LB liquid medium most strains displayed 25–30 min doubling times (τ); *sodA sodB* mutants grew slowly (τ = 40–60 min); *sodA sodB recA* mutants grew even more slowly (τ = 80–90 min, after several hours’ lag).

2.2. Chemicals and reagents

Bleomycin (Blenoxane®) was a gift from Bristol Laboratories. This metal-free sulfate mixture of bleomycins A₂ (60%), B₂ (30%), and other bleomycin congeners (10%) was dissolved in water and standardized optically as described [24]. Plumbagin, from Sigma Chemical Co. (St. Louis MO), was dissolved in 95% ethanol.

2.3. Bacterial susceptibility measurements

Susceptibility to the bacteriostatic action of plumbagin was estimated by applying 10 μ L of 40 mM plumbagin to 6 mm paper disks placed on agar plates onto which 0.05 mL of *E. coli* culture (10^9 cfu/mL) had been spread [25]. After overnight incubation at 37 °C, the zone of growth inhibition for each strain was measured — except for strain KD736 (*sodA sodB recA*), which took two days to grow discernibly.

For survival measurements, exponentially growing, aerobic bacterial cultures (~60 Klett units: $\sim 10^8$ cells/mL) received small volumes of bleomycin solutions with continued shaking at 37 °C. After 1/4, 4, 8, and 12 min exposure, aliquots were diluted 1,000-fold into ice-cold LB medium. Small volumes (3–600 μ L) of diluted cells were mixed with 3 mL of LB containing 0.6% agar (“top agar”) at 43 °C, applied immediately to drug-free LB agar plates, and incubated overnight for determination of surviving cells. Plumbagin was added to the initial cultures, when desired, in 1/40 vol of a 95% ethanol solution, together with any bleomycin; control cultures also received this ethanol treatment, which roughly doubled bleomycin killing potency without otherwise affecting cell viability (not shown). Percent survival was calculated relative

to colony-forming units present at the time of drug addition. First-order killing rates were calculated using all data points.

2.4. Superoxide dismutase assay

Specific activities of superoxide dismutase were assayed [26] in supernatant fluid (following 10 min centrifugation at 12,000 X g) of sonically disrupted concentrated cultures identical to those used intact in drug toxicity studies. These extracts contained about 1 mg protein per mL. For these incubations 60 μ M Nitro blue tetrazolium (Sigma Chemical Co., St. Louis MO), monitored by A₅₆₀, was reduced by superoxide produced continuously with 0.1 Unit/mL xanthine oxidase (EC 1.1.3.22, Boehringer, Germany), 150 μ M hypoxanthine (Fluka, Milwaukee, WI), and O₂. Added superoxide dismutase activity was assessed by its inhibition of this reduction. One unit of superoxide dismutase activity is the amount necessary to halve the reduction rate attributable to superoxide under these reaction conditions (1 mL reaction mixtures buffered with 20 mM Na-HEPES, pH 7.8, incubated at room temperature). Purified bovine erythrocyte superoxide dismutase (EC 1.15.1.1, Boehringer, Germany) served as an activity standard; an excess was added to confirm that >75% of the initial monitored Δ A₅₆₀ was due to superoxide. Protein was assayed with the Bio-Rad (Hercules, CA) Protein Assay Kit using bovine gamma globulin as the standard.

3. Results

3.1. Effect of intracellular superoxide dismutase on bleomycin lethality

E. coli has two superoxide dismutases (SODs), one inducibly expressed from the *sodA* locus (MnSOD) and the other constitutively expressed from *sodB* (FeSOD) [27,28]. To assess the effects of mutations, we measured the rate at which bleomycin killed *E. coli* at a variety of bleomycin concentrations. An example of this rate measurement is shown in Fig. 1A. In this experiment cells defective in both *sodA* and *sodB* were less susceptible to killing by bleomycin. When killing rates for various concentrations of bleomycin were compared, the *sodA sodB* mutant was less susceptible than wild-type cells, especially at higher bleomycin concentrations (Fig. 1B). The bleomycin congener phleomycin [29] showed the same effect as bleomycin, as did Cu(II)-bleomycin (not shown). Superoxide dismutase assays indicated that strains defective in both *sod* genes had SOD levels lowered by at least 6-fold (Table 2), and they presumably contained more superoxide.

With wild-type *E. coli*, adding a multi-copy plasmid (pDT1.5) that over-expressed the *sodA*⁺ gene increased superoxide dismutase 3-fold while another (pHS1.8), carrying *sodB*⁺, increased activity by 8-fold (Table 2). Similar superoxide dismutase activities were obtained with plasmid-bearing bacterial strains lacking an active chromosomal copy of *sodA* and *sodB* (strains KD617 and KD618, Table 2). The bleomycin susceptibility of SOD over-producer strain KD617 (*sodA sodB* pHS1.8) was greater than that of its *sod*-defective parent strain (QC774) or the wild-type strain (GC4468; Fig. 1). The SOD over-producer (strain KD617) was also hypersensitive to phleomycin and to Cu(II)-bleomycin (not shown). Thus, the lethal activity of bleomycin increased with increasing SOD, presumably due to the consequent decrease in superoxide concentration.

3.2. Effect of plumbagin-induced superoxide on bleomycin lethality

To elevate intracellular superoxide by metabolic means, the superoxide generator plumbagin [16] was added to cultures. Plumbagin treatment can itself be lethal, especially in strains lacking SOD ([16]; Table 2). Therefore, we chose plumbagin concentrations having relatively little lethality for each strain tested and examined their effects on bleomycin lethality. If killing by plumbagin and bleomycin were fully independent, their individual killing rates would be additive when they were administered together, but something very different occurred.

Plumbagin (0.5 mM) diminished bleomycin (0.04 mM) killing of wild-type *E. coli* by about 80% (Fig. 2A). This protective effect of plumbagin from bleomycin lethality was seen in several situations: with SOD mutants, with a SOD overproducer, for phleomycin, and for Cu(II)-bleomycin (Table 3). It appears that plumbagin, like *sod* mutations, may provide protection from bleomycin by increasing superoxide levels.

3.3. Effect of peroxide perturbations on bleomycin lethality

The two differing means used here to elevate intracellular superoxide provide a way to infer a possible role for peroxide, a product of superoxide dismutation. These differing methods of increasing superoxide should have opposite effects on transient intracellular peroxide levels: increasing superoxide formation (with plumbagin) should consequently increase peroxide formation, whereas increasing superoxide accumulation by decreasing its enzymatic dismutation (in *sodAB* strains) should decrease peroxide formation. The similar effects of both superoxide elevations on bleomycin lethality suggest that peroxide was not involved.

3.4. Effect of *recA* mutation on bleomycin lethality

Since DNA damage caused by bleomycin induces the SOS repair pathway [30] and since repair processes might be influenced by perturbation of superoxide [21], we repeated the plumbagin-rescue experiment of Fig. 2A with strains defective in *recA* and, thereby, in SOS repair. As expected, *recA56* strains were more sensitive to both plumbagin (Table 2) and bleomycin (Fig. 2). However, plumbagin was equally protective against bleomycin in otherwise isogenic *recA56* and *recA*⁺ strains (Fig. 2). The protective effect of plumbagin with *recA56* constructs was also observed with both the under- and overproducers of SOD (Table 3). Thus, the effect of superoxide on bleomycin toxicity appears to be independent of *recA*-mediated defense and repair pathways.

3.5. Effect of *sox* mutations on bleomycin lethality

A direct response to superoxide exposure is mediated by the superoxide-induced *soxR* regulon [21]. The presence of *sox* mutations failed to prevent plumbagin-mediated protection from lethal bleomycin challenge (Table 3). Protection was seen in both constitutive *soxR* strains tested (JTG1052 and JTG1078), in a Δ *sox* strain (JHC1092), and in a *soxQ* (*marA*) strain (JHC1071) having an elevated response to superoxide [21]. Thus the effect of plumbagin cannot be attributed solely to *sox* genes.

4. Discussion

It has been proposed that antibiotic-mediated lethality arises in part from the formation and then dismutation of superoxide, either spontaneously or enzymatically, to oxygen plus peroxide, which is then reduced by one electron to yield the highly toxic hydroxyl radical via the Fenton reaction [1,2,31]. The Fe(II) reductant for the Fenton reaction is thought to originate in cellular Fe-S clusters attacked by superoxide [1,2]. Support for this scenario includes the observations that 1) the lethal action of several antimicrobials is accompanied by increases in hydroxyl radical concentration [1,2], 2) mutations in genes encoding superoxide dismutase decrease the lethality of fluoroquinolones, β -lactams, and aminoglycosides [31], 3) mutations in catalase, an enzyme that converts peroxide to O₂ and water, increase the lethality of fluoroquinolones, β -lactams, and aminoglycosides [31], and 4) an iron chelator and a hydroxyl radical scavenger lower antimicrobial lethality [31].

In the present work we provided examples of protective effects of *sod* mutations and plumbagin against bleomycin. These effects indicate that increasing superoxide concentration protects from lethality by bleomycin (Fig. 1). Moreover, decreasing superoxide concentration by over-expressing a *sod* gene enhanced bleomycin lethality (Fig. 1), thereby complementing the *sod*

mutation findings. Augmenting superoxide production through treatment with plumbagin, which is expected to increase peroxide *via* superoxide dismutation, indicates that the buildup of superoxide can have a protective effect even when peroxide concentration is expected to increase (Table 3). Such results were expected neither from proposals that other lethal antimicrobials owe some of their lethality to the indirect generation of reactive oxygen species nor from superoxide's observed *in vitro* enhancement of bleomycin activation and consequent DNA cleaving activity, an activity that could enhance bleomycin-mediated cytotoxicity. The observation that the site specificity of *in vitro* and *in vivo* DNA cleavage is the same further reduces the likelihood that bleomycin cleaves DNA indirectly by initiating a reactive oxygen cascade [32].

The plumbagin versus bleomycin concentration effects have not been fully explored, and it is likely that complex relationships exist. For example, plumbagin concentrations that protected some strains from bleomycin lethality were toxic to others. Moreover, bleomycin susceptibility varied, which may, in turn, have affected which plumbagin concentrations were protective. However, every strain tested showed plumbagin-mediated protection from bleomycin at some concentrations of the two (Table 3).

The mechanism of superoxide's protective effect is not yet known. One possibility is that superoxide stimulates a self-destructive reaction of bleomycin. The formation of activated bleomycin, which is stimulated by superoxide, leads to the production of degraded, inactive antibiotic when DNA is absent [33]. If this process occurs in the cellular periphery, it could lower the concentration of intact bleomycin reaching its DNA target. A less likely scenario is a superoxide attack of iron-sulfur clusters that releases Fe ions [34]; less likely because increased Fe availability is expected to enhance bleomycin activity rather than inhibit it. Indeed, addition of the Fe(II) chelator desferrioxamine to bacterial cultures protects from bleomycin-mediated cell death (not shown). A very different possibility is that superoxide elicits a protective cellular response that over-shadows any reactive oxygen cascade proposed to be associated with lethal antibiotics [2,31]. Such a protective response is unlikely to involve RecA activity because a *recA* deficiency showed no effect (Fig. 2). Likewise, no obvious effect was seen with *sox* mutations (Table 3), although more subtle effects on the interplay of bleomycin and superoxide may have been overlooked. We are now determining whether plumbagin protects *E. coli* from other types of antimicrobial stress, since a general effect would help distinguish bleomycin-specific chemical events from cellular responses.

Abbreviations

BLM, bleomycin; HEPES, N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonate; LB, Luria Broth [17] bacterial culture medium; SOD, superoxide dismutase.

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Fig. 1. Lethal action of bleomycin on *E. coli* strains having different levels of superoxide dismutase. Exponentially growing cultures for panel A were exposed to 10 μM bleomycin for the indicated times, and percent survival (determined in triplicate) is shown relative to an untreated culture at the time of bleomycin addition. Symbols: wild type (GC4468, open circles), *sodA sodB* (QC774, open triangles), *sodA sodB* plus plasmid-borne *sodB*⁺ (KD617, filled circles), wild type plus plasmid-borne *sodA*⁺ (KD594, filled triangles). Panel B shows the dependence of killing rate on bleomycin concentration with three of the same *E. coli* strains, as in panel A (same symbols). First order killing rates (min⁻¹) were determined from progress curves including those shown in panel A.

Fig. 2.

Effect of plumbagin on bleomycin lethality. Exponentially growing cultures of wild-type cells (GC4468, SOD 0.7 U/mg protein, panel A) or a *recA56* mutant (KD735, SOD 0.8 U/mg protein, panel B) were exposed for the indicated times to either 0.5 mM plumbagin (PLB, open circles), bleomycin (BLM, filled triangles: 40 μ M for GC4468, 10 μ M for KD735), or both plumbagin and bleomycin (filled circles). Viable counts were from triplicate measurements; replicate experiments gave the same results.

Scheme 1.

Roles of dioxygen species in activating Fe-bleomycin (Fe-BLM) *in vitro*. An additional O_2 is required to resolve DNA damage to deoxyribose cleavage (not shown; reviewed in [6]). It is not known which pathways are important *in vivo*.

Table 1**Bacterial strains and plasmids**

Strain or Plasmid number	Relevant genotype	Source or Reference
GC4468	wild type K-12	D. Touati [18]
JC10240	<i>recA56</i>	A.J. Clark [19]
JHC1071	GC4468 <i>soxQI</i> ^a	B. Demple [20,21]
JHC1092	GC4468 Δ <i>soxR</i>	B. Demple [21]
JTG1052	GC4468 <i>soxR10I</i> ^c	B. Demple [21]
JTG1078	GC4468 <i>soxR105</i> ^c	B. Demple [21]
KD594	GC4468 pDT1.5	This work by transformation
KD595	GC4468 pHS1.8	This work by transformation
KD617	QC774 pDT1.5	This work by transformation
KD618	QC779 pHS1.8	This work by transformation
KD735	GC4468 <i>recA56</i>	This work by P1-transduction from JC10240
KD736	QC774 <i>recA56</i>	This work by P1-transduction from JC10240
KD737	KD594 <i>recA56</i>	This work by P1-transduction from JC10240
pDT1.5	<i>sodA</i> ⁺ plasmid	D. Touati [18,22]
pHS1.8	<i>sodB</i> ⁺ plasmid	D. Touati [18,22,23]
QC774	GC4468 <i>sodA sodB</i>	D. Touati [18]
QC779	GC4468 <i>sodA sodB</i>	D. Touati [18]

^a Superoxide-inducible over-expression of the *sox* operon. *SoxQ* is now known as *marA* [20].

^c Constitutive phenotype.

Table 2

Levels of superoxide dismutase and of plumbagin susceptibility

Strain number	Relevant genotype	Superoxide Dismutase (U/mg protein)	Plumbagin Susceptibility ^a (mm inhibition)
GC4468	wild type	0.7	6
QC774	<i>sodA sodB</i>	< 0.1	23
QC779	<i>sodA sodB</i>	< 0.1	21
KD594	<i>sodA</i> ⁺ plasmid	2.4	6
KD595	<i>sodB</i> ⁺ plasmid	5.7	6
KD617	<i>sodA sodB sodA</i> ⁺ plasmid	3.6	6
KD618	<i>sodA sodB sodB</i> ⁺ plasmid	5.6	6
KD735	<i>recA56</i>	0.8	13
KD736	<i>sodA sodB recA56</i>	< 0.1	70
KD737	<i>recA56 sodA</i> ⁺ plasmid	1.7	6

^a Diameter of inhibition around a 6 mm paper disk with 10 μ L of 40 mM plumbagin: 6 mm signifies no inhibition.

Table 3

Plumbagin antagonism of bleomycin lethality to oxidant-protection variants

Strain	Relevant genotype	Plumbagin (mM)	Killing rate ^a min ⁻¹ with Bleomycin	b	Rate ratio ^c (μM) Both c/b ^d
GC4468 wild type		0	(0.5) 0.44		(40) 0.12 0.27
GC4468 wild type		0	(0.5) 0.69		(Phleo. ^d 3) 0.23 0.33
GC4468 wild type		0	(0.5) 0.69		(CuBLM 40) 0.06 0.09
JHC1071 <i>soxQ1</i> (<i>marA</i>)		0	(0.5) 0.45		(20) 0.24 0.52
JHC1092 <i>dsxR</i>		0.06	(0.5) 0.47		(10) 0.06 0.13
JTG1052 <i>soxR101</i> ^c		0	(0.5) 0.52		(20) 0.26 0.51
JTG1078 <i>soxR105</i> ^c		0	(0.5) 0.23		(20) 0.11 0.49
KD594 <i>sodA</i> ⁺ plasmid		0.11	(0.5) 0.63		(20) 0.22 0.34
KD735 <i>recA56</i>		0.04	(0.5) 0.49		(10) 0.12 0.25
KD736 <i>recA56 sodA sodB</i>		0.04	(0.5) 0.58		(10) 0.04 0.07
KD737 <i>recA56 sodA</i> ⁺ plasmid		0	(0.5) 0.86		(10) 0.30 0.35
QC779 <i>sodA sodB</i>		0.09	(0.3) 0.29		(50) 0.08 0.30

^aFirst order killing rates were measured in experiments like those of Fig. 1 and Fig. 2.

^bThe ratio of rates, c/b, measures plumbagin's antagonism of bleomycin lethality, ranging from ≥1 = no protection, to 0 = complete protection.

^cDesignates a constitutive phenotype.

^dAbbreviations used are: Phleo., Phleomycin; CuBLM, a pre-formed Cu(II)-BLM complex, each used instead of bleomycin.