Cell Death from Antibiotics Without the Involvement of Reactive Oxygen Species

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Recent observations have suggested that classic antibiotics kill bacteria by stimulating the formation of reactive oxygen species (ROS). If true, this notion might guide new strategies to improve antibiotic efficacy. In this study, the model was directly tested. Contrary to the hypothesis, antibiotic treatment did not accelerate the formation of hydroxyl peroxide in Escherichia coli and did not elevate intracellular free iron, an essential reactant for the production of ROS. We conclude that these antibiotic exposures did not produce ROS and that lethality more likely resulted from the direct inhibition of cell-wall assembly, protein synthesis, and DNA replication.

In recent decades, the growing number of antibiotic-resistant pathogens has spurred efforts to further understand and improve the efficacy of the basic antibiotic classes. Most clinically used antibiotics target cell-wall assembly, protein synthesis, or DNA replication. However, recent reports have raised the possibility that although these antibiotics block growth by directly inhibiting the targets mentioned above, they may owe their lethal effects to the indirect creation of reactive oxygen species (ROS) that then damage bacterial DNA (1–10).

The evidence supporting this proposal included the observation that cell-penetrating dyes were oxidized more quickly inside antibiotic-treated bacteria (3–8). Furthermore, iron chelators (3, 4, 7–9), which suppress hydroxyl-radical–generating Fenton chemistry, and thiourea (4, 6, 8–10), a potential scavenger of hydroxyl radicals, lessened toxicity. Mutations that diminish fluxes through the tri-carboxylic acid cycle were protective (3–5), suggesting a key role for respiration, and DNA-repair mutants were somewhat sensitive (4, 8). Systems analysis of aminoglycoside-treated Escherichia coli suggested a model that fits these data (5). It was postulated that interference with ribosome progression would release incomplete polypeptides, some of which are translocated to the cell membranes where they might trigger envelope stress. The Arc regulatory system is perturbed, potentially accelerating respiration and thereby increasing the flux of superoxide and hydrogen peroxide into the cell interior. These two oxidants have known sequelae that ultimately lead to DNA damage. Specifically, superoxide and hydrogen peroxide damage the iron-sulfur clusters of dehydratases (II, 12), releasing iron atoms and elevating the pool of intracellular unincorporated iron (13, 14). This iron can then react with hydrogen peroxide in the Fenton reaction, generating hydroxyl radicals that either directly damage DNA (15) or indirectly oxidize the deoxynucleotide pool, which is subsequently incorporated into DNA (16). This scenario could explain the observed oxidation of intracellular dyes, protection by scavengers and chelators, a requirement for respiration, and the sensitivity of DNA-repair mutants.

This model is plausible, so we devised experiments to directly test the molecular events that underpin it. The bacterial strain (E. coli MG1655), growth medium (LB), and antibiotic doses were chosen to match those of previous studies (4). Kanamycin was used to target translation, ampicillin to block cell-wall synthesis, and norfloxacin to disrupt DNA replication.

Superoxide and hydrogen peroxide are generated inside cells when flavoenzymes inadvertently transfer a fraction of their electron flux directly to molecular oxygen (15, 17). Thus, neither of these ROS can be formed under anoxic conditions.

**Fig. 1.** Antibiotic efficacy does not require oxygen or H₂O₂. (A to C) Wild-type cells were treated with ampicillin (A), norfloxacin (B), or kanamycin (C) in the presence (solid squares) or absence (open squares) of oxygen. In (C), anoxic killing was also tested in the presence of 40 mM KNO₃ (gray squares). (D to F) Wild-type cells (solid squares, MG1655) and congenic Hpx⁺ cells (open diamonds, AL427) were treated with antibiotics ampicillin (D), norfloxacin (E), or kanamycin (F). Results are representative of at least three biological replicates.
conditions. We found, however, that ampicillin and norfloxacin were as lethal to cells in an anaerobic chamber as to cells in air-saturated medium (Fig. 1, A to C). Anoxia sharply diminished the toxicity of kanamycin, but lethal activity was partially restored at high kanamycin doses when nitrate was provided as an alternative respiratory substrate. This pattern mirrors the effects of oxygen and nitrate upon kanamycin import, which is governed by the magnitude of the proton motive force (18, 19). Thus, ROS were not required for the lethal actions of kanamycin, and they made no apparent contribution at all to the toxicity of ampicillin and norfloxacin.

Hydroxyl radicals are formed inside cells when ferrous iron transfers electrons to H$_2$O$_2$, so hydroxyl-radical stress is greatest when either H$_2$O$_2$ or ferrous iron transfers electrons to H$_2$O$_2$. Hydroxyl radicals are formed inside cells when ferrous iron transfers electrons to H$_2$O$_2$, so hydroxyl-radical stress is greatest when either H$_2$O$_2$ or ferrous iron transfers electrons to H$_2$O$_2$.

An E. coli mutant that lacks catalase and peroxidase activities (denoted Hpx*) cannot scavenge H$_2$O$_2$ and more easily accumulates it to toxic levels; substantial damage to proteins and to DNA ensues (12, 20, 21). However, this mutant was not more sensitive to ampicillin or kanamycin than were wild-type cells (Fig. 1, D and F). It was more sensitive to norfloxacin, an effect that might result from the preexisting DNA damage in the scavenger mutant, coupled with the inhibitory effect of norfloxacin on DNA metabolism (22) (Fig. 1E).

We directly tested the prediction that antibiotic treatment would augment respiration and thereby increase superoxide and H$_2$O$_2$ formation. The respiratory chain is normally a minor source (<20%) of these oxidants (17), so a very large flux increase would be needed to substantially amplify total intracellular oxidant production. However, measurements of oxygen consumption with a Clark-type electrode showed that respiration slowly declined, rather than increased, when cells were treated with kanamycin (Fig. 2A). This trend persisted throughout the period during which the number of viable cells declined by >99%. Norfloxacin had little effect on respiration. Ampicillin transiently increased respiration, perhaps by causing envelope damage that dissipated the back-pressure of the proton motive force, before cell lysis ended metabolism. In no case did respiration accelerate enough to be a sufficient explanation for toxicity.

E. coli features a transcription factor, OxyR, that is activated by H$_2$O$_2$ whenever its levels approach the concentrations (0.5 to 1 μM) sufficient to damage enzymes or DNA (23). The OxyR regulon includes genes whose products assist in H$_2$O$_2$ scavenging (katG and ahpCF), free-living control (dps, fur, and yaaA), and enzyme protection (mntH and sufABCDE) (15, 24). When cells were treated with ampicillin and norfloxacin, they accumulated lethal damage within 1 hour; however, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis indicated that OxyR-controlled genes were not induced (Fig. 2B). These antibiotic-treated cells robustly activated katG, ahpC, and yaaA when exogenous H$_2$O$_2$ was added as a control. (After 1 hour, kanamycin-treated cells failed to respond even to authentic H$_2$O$_2$, perhaps because OxyR protein was depleted in these translationally blocked cells.) This result was confirmed by measurements of katG::lacZ expression (Fig. 2, C and D). Thus, neither ampicillin nor norfloxacin create enough H$_2$O$_2$ stress to trigger the natural H$_2$O$_2$ sensor within the cell.

The rate of intracellular H$_2$O$_2$ formation can be directly measured using catalase/peroxidase mutants. Endogenous H$_2$O$_2$ rapidly diffuses from these cells out into the growth medium, and the rate of its accumulation can be quantified by direct assay (Fig. S1) (17). When cells were treated with lethal concentrations of kanamycin and ampicillin, H$_2$O$_2$ production did not increase (Fig. 2, E to H). In norfloxacin-treated cells H$_2$O$_2$ formation increased slightly (~2-fold) after 1 hour but not at all within the first 30 min when >99% of cells accumulated lethal damage. In contrast, when cells were treated with a nonlethal dose of the redox-cycling drug paraquat, H$_2$O$_2$ production was stimulated ~7-fold. We conclude that these classic bactericidal antibiotics did not promote H$_2$O$_2$ formation.

There is one other way that hydroxyl-radical formation might be accelerated: by increases in the amount of unincorporated iron. Iron can accumulate inside cells under conditions of superoxide stress, due to the destruction of labile enzymic iron-sulfur clusters (13, 14), and this mechanism was postulated to occur during antibiotic exposure (4). We monitored the status of 6-phosphogluconate dehydrogenase, an iron-sulfur

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**Fig. 2.** Antibiotics do not create H$_2$O$_2$ stress. (A) O$_2$ consumption rates were not substantially elevated by antibiotic treatment. Oxygen consumption by wild-type cells was measured with a Clark-type electrode before and during exposure to 5 μg/ml kanamycin, 5 μg/ml ampicillin, or 250 ng/ml norfloxacin. Respiration rates were normalized to optical density. (B) The OxyR regulon was not induced by ampicillin (5 μg/ml) or norfloxacin (250 ng/ml). mRNA was collected from naive and antibiotic-exposed wild-type cells, and the expression of OxyR-responsive genes was quantified by qRT-PCR. Signals were normalized to that of the housekeeping gene gapA, and fold inductions were calculated. Where indicated, cultures were additionally treated with exogenous H$_2$O$_2$ at room temperature for 10 min before mRNA collection. (C and D) Expression of a katG::lacZ fusion was measured in exponential-phase wild-type cells (AL441) before and after antibiotic incubations (C). As a positive control, expression was measured in wild-type and Hpx* cells (AL494) before (+O$_2$) and after (−O$_2$) 1-hour aerations (D). (E to H) Classic antibiotics—ampicillin (E), kanamycin (F), and norfloxacin (G)—did not promote H$_2$O$_2$ formation. Aerobic Hpx* cells (AL427) were treated with antibiotic, and at designated time points samples were collected and the ongoing rate of H$_2$O$_2$ production was measured. The redox-cycling compound paraquat (H) was included as a positive control; this dose was insufficient to cause cell death (viable counts increased for ~3 hours).

Data in this figure are reported as the means and SEM from at least three independent experiments.
of oxidative damage—enzyme damage, increases in free-iron levels, or hypersensitivity of DNA-repair mutants—were detected.

Fluorescein-based dyes such as hydroxy-phenyl fluorescein are more quickly oxidized within antibiotic-treated cells than in control cells, and this fact has been regarded as evidence that hydroxyl radicals are present (25). Indeed, thiourea, a presumptive scavenger of hydroxyl radicals, diminished the antibiotic-augmented fluorescence. However, we observed that although thiourea efficiently blocked dye oxidation in a simple in vitro Fenton system, ethanol—another hydroxyl-radical scavenger of similar efficiency (26)—failed to do so, even at far higher doses (fig. S5, A and B). This result implies that the dye was actually oxidized by the high-valence iron ($\text{FeO}^{3+}$) initially formed by the Fenton reaction, rather than by the hydroxyl radical to which it decomposes; thiourea, a sulfur compound, may have re-reduced the metal before it oxidized the dye. Indeed, the dye was very rapidly oxidized by horseradish peroxidase (fig. S5C), which has an analogous high-valence metal intermediate but does not release hydroxyl radicals (27). The dye was even oxidized by ferricyanide, a ferric-iron chelate of moderate oxidizing potential (+0.44 V) (fig. S5E).

DNA is substantially damaged when hydroxyl radicals are rapidly formed inside cells, and mutants that are deficient in the excision or recombinational repair of oxidative DNA lesions are particularly vulnerable (15). The extreme sensitivity of recA and polA mutants to exogenous H$_2$O$_2$ is illustrated in fig. S3A. However, recA strains exhibited little or no hypersensitivity to killing doses of kanamycin or ampicillin, suggesting that these antibiotics have little effect upon killing doses of kanamycin or ampicillin, suggesting that these antibiotics have little effect upon these antibiotics do not introduce lethal damage (14). These levels did not rise in kanamycin- or norfloxacin-treated cells (Fig. 3, D and E). (Levels could not be measured after ampicillin treatment, as lysing cells could not be concentrated for the measurement.) In contrast, free-iron levels were increased 4-fold in fur regulatory mutants that oversynthesize iron import systems (Fig. 3F). Notably, although these fur mutants were slightly more sensitive to ampicillin, they were actually more resistant to kanamycin and norfloxacin (fig. S2). This result, also observed by others (3), indicates that intracellular free-iron levels do not correlate with antibiotic sensitivity.

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Other workers noted that lower doses (1 to 2 µg/ml) of ampicillin slowly kill recA mutants while being merely bacteriostatic to wild-type strains (4, 8). We reproduced that result (fig. S4A). However, this behavior also occurred under anoxic conditions, when ROS are nonexistent (fig. S4B). The slight sensitivity of recA mutants may reflect defects in septation from their innate replication problems. At higher doses of ampicillin, i.e., at doses sufficient to kill wild-type bacteria, recombination efficiency had little or no impact on death rate.

Collectively, these data indicate that the known mechanisms of oxidative stress by ROS were not involved in causing the death of antibiotic-treated cells. Oxygen was dispensable for toxicity, and neither OxyR activity nor direct measurements of H$_2$O$_2$ formation indicated that ROS were formed at accelerated rates. Further, no customary markers of oxidative damage—enzyme damage, increases in free-iron levels, or hypersensitivity of DNA-repair mutants—were detected.

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Finally, the fluorescence of the oxidized dye

![Fig. 3.](https://www.sciencemag.org)
product was itself quenched after the fact by addition of the same doses of thiourea that were used in antibiotic studies (fig. S5D); this effect cannot be due to hydroxyl-radical scavenging activity. Thus, it is plausible that fluorescein-based dyes are predominantly oxidized in vivo by oxidized enzyme metal centers, which may be more prominent in antibiotic-treated cells as metabolism fails. In any case, one must be cautious in interpreting the oxidation of these dyes inside cells.

Finally, cell protection by either thiourea or iron chelators cannot be regarded as diagnostic of lethal Fenton chemistry. Both agents suppressed antibiotic sensitivity even under anaerobic conditions (fig. S6), when death occurs through pathways that do not involve ROS. We conjecture that they did so by reducing growth and/or metabolic rates. In general, it is unlikely that exogenous agents like thiourea could substantially diminish the lifetimes of hydroxyl radicals in vivo, because these radicals already react at nearly diffusion-limited rates with intracellular biomolecules that are collectively at molar concentrations (28). Iron chelators have pleiotropic effects, including the repression of tricarboxylic acid–cycle and respiratory enzymes (29), and may thereby diminish the membrane potential that drives aminoglycoside uptake. Synthesis of these enzymes is also repressed when cells are fed glucose, and this treatment analogously diminished kanamycin sensitivity (fig. S7). Thus, it is advisable to follow up experiments that rely upon these agents with ones that employ more direct markers of oxidative stress.

We conclude that under our experimental conditions, these major classes of antibiotics did not exert their lethal actions through the known mechanisms of oxidative stress. Of course, this does not preclude the possibility that antibiotics trigger mechanisms of stress that do not involve the ROS that were tested here.

References and Notes

Killing by Bactericidal Antibiotics Does Not Depend on Reactive Oxygen Species

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Bactericidal antibiotics kill by modulating their respective targets. This traditional view has been challenged by studies that propose an alternative, unified mechanism of killing, whereby toxic reactive oxygen species (ROS) are produced in the presence of antibiotics. We found no correlation between an individual cell’s probability of survival in the presence of antibiotic and its level of ROS. An ROS quencher, thiourea, protected cells from antibiotics present at low concentrations, but the effect was observed under anaerobic conditions as well. There was essentially no difference in survival of bacteria treated with various antibiotics under aerobic or anaerobic conditions. This suggests that ROS do not play a role in killing of bacterial pathogens by antibiotics.

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