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Point of VIEW

Bactericidal Antibiotics and Oxidative Stress: A Radical Proposal

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ABSTRACT A recent publication suggests that many antibiotics exert their bactericidal effects *via* the production of hydroxyl radicals, regardless of their molecular targets. This proposal represents an abrupt departure from conventional models, and it will attract further experimental tests.

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A paper by Kohanski *et al.* in the September 7 issue of *Cell* (*1*) presented evidence that bactericidal antibiotics kill both representative Gram-negative (*e.g.*, *Escherichia coli*) and Gram-positive (*e.g.*, *Staphylococcus aureus*) bacteria by an as yet unappreciated mode: through overproduction of the destructive oxygen reduction product, the hydroxyl radical ($HO^{\cdot}$). The three bactericidal antibiotics used in the study were ampicillin (a β-lactam), kanamycin (an aminoglycoside), and norfloxacin (a quinolone). These antibiotics target peptidoglycan biosynthesis, protein synthesis, and DNA replication machinery, respectively. Despite the diversity of their actions, these compounds each stimulated the intracellular oxidation of hydroxyphenyl fluorescein, a compound that is used to probe the rate of hydroxyl radical formation. Further, the toxicity of the antibiotics was substantially diminished by both a cell-penetrating iron chelator (which blocks the Fenton reaction that generates hydroxyl radicals) and a putative hydroxyl radical scavenger. Other metabolic factors that might influence the rate of hydroxyl-radical generation, including TCA cycle function, NADH levels, and iron–sulfur cluster assembly, also affected the rate of cell killing. The authors proposed that antibiotics somehow stimulate cell respiration, with a consequent acceleration of the endogenous formation of reactive oxygen species (ROS). The sequelae of intracellular hydrogen peroxide and superoxide formation are well-established: these species can destroy enzymic iron–sulfur clusters, and iron atoms that are thereby released can reduce hydrogen peroxide and generate hydroxyl radicals. The consequent oxidation of DNA can be a lethal event.

The challenge for investigators now is to probe the details of the scheme, which includes some mechanistic gaps. Further, it will be important to appraise whether induced oxidative stress would have a substantial role in antibiotic action in real-world infections, as bacterial growth occurs most frequently in oxygen-limited biofilms rather than in planktonic (free-living) cultures.

How Might Antibiotics Create Oxidative Stress? Because no direct method is available to quantify intracellular levels of ROS *in vivo*, much of the research community has turned to the use of derivatized fluoresceins as a semiquantitative measure. In their reduced forms, these compounds can gradually penetrate cells, where their oxidation can convert them to a fluorescent specie that can be detected by microscopy or cell sorting. The latter approach was used in the Kohanski study, and experiments showed that antibiotic-treated cells accumulated fluorescent hydroxyphenyl fluorescein at concentrations up to an order of magnitude higher than nontreated cells. The authors suggest that hydroxyl radicals were the oxidant, an idea that was supported by the ability of an iron chelator and thiourea to suppress the effect. Indeed, the iron chela-


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tor dipyridyl has been shown to block the Fenton reaction *in vivo* without affecting the concentrations of other oxygen species. The thiourea effect is not so easily understood, however, as exogenous hydroxyl radicals scavengers should not significantly affect the lifetime of the hydroxyl radical inside cells, where there are molar concentrations of other biomolecules with which it can rapidly react.

Does the elevated fluorescence indicate that hydroxyl radicals are rapidly generated inside antibiotic-treated cells? In experiments of this type, an unambiguous interpretation is precluded by the possibility that antibiotic treatment facilitates the entry of the fluorescein into the dying cells or else that the concentrations of biomolecules that might otherwise quench the fluorescence are diminished. Nevertheless, the authors' interpretation is plausible and raises the question of how antibiotic treatment might generate oxidative stress. Kohanski *et al.* tentatively suggest that antibiotic treatment might somehow promote an acceleration of respiration and that hydrogen peroxide, a precursor to the hydroxyl radical, is generated as a toxic byproduct. It is not clear how this would happen; no linkage between antibiotic action and respiratory control is known. Further, in both bacterial and mammalian systems, respiratory acceleration actually correlates with a diminution of ROS formation, since it depletes electrons from the autoxidizable components of the electron transport chain. In any case, this step of the model is testable, since methods are in hand to measure both the metabolic rate and the respiratory activity of intact cells.

The heart of the matter, though, is whether ROS levels rise by any mechanism. Several alternative markers of oxidative stress are available that might complement the fluorescein data: ROS diminish the activities of acutely oxidant-sensitive enzymes, and they induce well-studied genetic regulons. Substantially <1 μM of intracellular hydrogen peroxide is sufficient to activate OxyR-controlled genes (*2, 3*), and one would certainly expect this regulon to be activated by doses that were capable of killing the cell. Similarly, the SoxRS system responds to a wide variety of drugs that generate ROS through redox cycling (*4–6*). The authors did not find oxidant-responsive genes among those that were induced by the tested antibiotics; nevertheless, there are a variety of reasons why negative results are sometimes obtained, and the specific probes can be used to resolve this question unambiguously.

A final issue that warrants attention is whether oxidative stress is a source or a consequence of cell damage. A precedent exists: previous work in mammalian systems revealed that lipid peroxidation, once thought to be a driver of cell death during a wide variety of stresses, instead ensues from the progressive failure of antioxidant systems as the cells die from other causes (*7*). In the current study, Kohanski *et al.* found that iron chelators and metabolic mutations were protective against the antibiotics, which suggests that the oxidants directly created the lethal injuries. However, the authors also acknowledge that the chelators and mutations also slow growth, and since antibiotics are generally more effective against cells that grow quickly, this clouds the issue of cause and effect. Dissection of these connections will require further work. One approach would be to examine bacterial mutants that lack superoxide dismutase and/or catalases and peroxidases: if the central premise of the study is correct, then these strains should be hypersensitive to bactericidal antibiotics.

Does This Experimental System Reflect the Mechanism of Antibiotic Action in Natural Environments? These studies were performed under aerobic, planktonic conditions. In contrast, >70% of bacterial infections are composed of biofilms, highly organized bacterial communities that are typically encased in a polysaccharide and/or proteinaceous matrix (*8*). This difference can affect fundamental bacterial physiology, in part because cells located at different positions in a biofilm differ in growth rate and antibiotic exposure. Of particular significance to the current issue is the fact that the biofilm can create an oxygen-poor environment. In these habitats, pathogenic bacteria such as *E. coli* or *S. aureus* maintain their energy charge by using high-affinity cytochrome oxidases, through anaerobic (nitrate-dependent) respiration, or by fermentation. Since ROS are generated in direct proportion to oxygen concentration, it is likely that in biofilms the intracellular production of ROS must be minimal or nonexistent. Yet bactericidal antibiotics, including those used in the Kohanski investigation, remain effective in these conditions, indicating that oxidative injury must not be their sole mechanism of killing. The same is true in absolutely anaerobic laboratory cultures. The discrepancy between the anaerobic efficacy of antibiotics and their apparent dependence upon oxidative mechanisms in this study might stem from the concentrations of antibiotics that were used, which were lower than the levels desired in blood, urine, and cerebrospinal fluid. Thus, oxidative injuries might contribute to toxicity, but they clearly cannot be essential.

Conclusions. A connection between bactericidal antibiotics and oxidative stress was

unanticipated and is exciting. Kohanski *et al.* point out that if this mechanism is true, then one might be able to enhance antibiotic efficacy by parallel treatments that compromise the oxidative defenses of the bacteria. Such a tactic is not out of reach, since over the past two decades intense investigation has revealed many of the strategies by which bacteria defend themselves against oxidants. At this point, follow-up work is merited to verify the tentative conclusions of the Kohanski study, as well as to fill in the gaps in their proposed mechanism. In any case, the results of this paper indicate that the effective killing of infectious bacteria by bactericidal antibiotics is almost certainly more complicated than is currently recognized.

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