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COMMENTARY

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Interpreting the role of antioxidants in vivo: A cautionary tale

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Abstract

Bacteria have a remarkable ability to sense environmental stresses and to respond to these stressors by adapting their metabolism and physiology. In recent publications, investigators have suggested that multiple stresses that cause cell death share the mechanistic feature of stimulating the formation of reactive oxygen species (ROS). A central piece of evidence cited in these claims is the ability of exogenous antioxidant compounds to mitigate stress-related cell death. The validity of attributing a positive effect of exogenous antioxidants to ROS-mediated stress is challenged by an important study by Korshunov and Imlay in this issue of *Molecular Microbiology*. This study reports biochemical data that convincingly show that some commonly used antioxidants quench oxidants orders of magnitude too slowly to have a significant effect on the concentration of ROS in the cell. Under conditions where antioxidants minimize cell death, they also slow growth. Significantly, slowing cell growth by other means has the same restorative effect as adding an antioxidant. Based on the solid biochemical and genetic data, Korshunov and Imlay make the case for discarding the use of antioxidants to diagnose conditions that generate increased internal ROS production.

KEYWORDS
antioxidants, reactive oxygen species

Microbes inhabit and thrive in all environmental niches on the planet. The ability to populate such a wide variety of environments demands the evolution of metabolic and physiologic capabilities that allow microorganisms to effectively utilize resources available in their environments. Many environments contain elements that are toxic or stressful to their inhabitants, and thus populating these niches requires the evolution of stress responses and/or the means to mitigate damage by stressors. In addition to stresses applied externally by the environment, organisms must deal with stressors that are internally generated by the metabolic strategies they use. Endogenous stressors include reactive metabolites generated as pathway intermediates, produced as side reactions in metabolic reactions, or from "moonlighting" activity of enzymes (Borchert et al., 2019; Caranto & Lancaster, 2017; Imlay, 2013; Kim & Copley, 2012).

Perhaps the best-known example of endogenously generated stressors is reactive oxygen species (ROS) (Imlay, 2013). These chemical species include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$). Superoxide and hydrogen peroxide are formed when molecular oxygen adventitiously collides with the reduced flavins and metal centers of redox enzymes (Imlay, 2013). The hydroxyl radical ($OH^\cdot$) can react strongly with both organic and inorganic molecules including DNA and proteins. Generation of and exposure to ROS is an unavoidable consequence of life in toxic environments. The potential damage caused by ROS is the cost of enjoying the benefits an oxic lifestyle confers.

Beyond endogenously generated ROS, there are redox-cycling compounds that can accelerate internal ROS formation when provided exogenously. Included in this class of compounds are

antibiotics produced by various bacteria, fungi and plants, and artificial redox-cycling agents, such as paraquat (de Paiva et al., 2003; Hassan & Fridovich, 1979; Inbaraj & Chignell, 2004; Turner & Messenger, 1986). These redox-cycling compounds undergo intracellular redox transformations and thus cause toxicity. These agents impair cell function by removing electrons from carriers and redirecting them to new targets, disrupting normal electron flow and metabolism. Collectively the generation of, and the damage by, ROS has been studied over decades and is well understood in bacterial model systems (Imlay, 2013). It is significant that, in general, ROS are not lethal, but rather paralyze growth by damaging [4Fe-4S] clusters or Fe (II) cofactors of critical enzymes, and by oxidizing DNA. Bacteria typically wield an array of scavenging enzymes to defend against ROS, and they also have enzymes that repair cellular damage caused by ROS. As one might expect, the protective mechanisms used by *Escherichia coli* and other bacteria are well understood. The primary line of defense is enzymes such as superoxide dismutases (SOD, which remove superoxide anion) and peroxidases and catalases (which destroy hydrogen peroxide and other peroxides).

In principle, increased ROS could overcome the capacity of the scavenging enzymes and cause cell death. In fact, this premise has been applied to explain the toxicity of several conditions/agents that cause cell death, ranging from thermal shocks to antibiotic treatments (e.g., Hong et al., 2020; Hwang & Lim, 2015; Kaur et al., 2017; Marcén et al., 2017; Ramakrishnan et al., 2016; Rodríguez-Rosado et al., 2019; Shekhova et al., 2017). In general, the hypothesis that links these studies is that increased ROS are generated or accumulated during treatment with a stressor. As Korshunov and Imlay point out, the number and diversity of stresses that have been tentatively linked to ROS is overwhelming. The direct targets of these stresses, when known, are varied making it difficult to envision a mechanism by which each of them could increase ROS formation.

A key piece of evidence that is purported to link different stressors with ROS in numerous studies is the demonstration that exogenous chemical antioxidants increase cell survival. The relevant antioxidants are organic compounds that quench O_2^-, H_2O_2, or hydroxyl radicals and are thereby presumed to protect important endogenous biomolecules. Commonly used antioxidants are thiourea, N-acetylcysteine, glutathione, and ascorbic acid. Each of these molecules shows detectable ROS-degrading activity in vitro. Yet, the notion that if antioxidants quench ROS in vitro, they can/will similarly quench them in vivo relies on the assumption that events that occur in a test tube adequately mimic the cellular milieu. Korshunov and Imlay meticulously test this assumption in the context of ROS degradation and conclude that antioxidants are ineffective inside cells that have a full cadre of scavenging enzymes (Korshunov & Imlay, 2024). Thus, the conclusion that cell death is caused by ROS simply based on the ability of antioxidants to increase cell survival is misleading.

Korshunov and Imlay confirm that thiourea, glutathione, N-acetylcysteine, and ascorbate quench superoxide and hydrogen peroxide in vitro. Rate constants for the reactions with each antioxidant and the two ROS were determined. Using these rate constants, the impact that each antioxidant molecule would have on superoxide and hydrogen peroxide concentrations in the *E. coli* cell was calculated. To reduce the steady state of superoxide by two-fold, the scavenging activity of the antioxidant would have to equal that of endogenous SOD activity. To lower the superoxide concentration in the cell to this level, a four to 99 M concentration of antioxidant would be required! Similar calculations considering H_2O_2 and the scavenging activity of catalase found that antioxidant concentrations ranging from four to 16 M would be required to achieve a two-fold reduction in H_2O_2 concentration. It is not possible to reach these concentrations in the cell, even with exogenous supplementation. Consider that thiourea is commonly used as an antioxidant at 150 mM. Such a concentration would reduce the steady state of superoxide *by only 0.15%*, and then only if the internal concentration matched the exogenous one. Thus, the calculations based on in vitro results and assays of endogenous enzyme activity convincingly showed that chemical antioxidants would not significantly impact the concentration of ROS the cell. In total, the authors showed that the rate of ROS removal in vitro is orders of magnitude too slow for chemical antioxidants to have an impact on the in vivo concentrations of superoxide anions and peroxides. Instead, the cell relies on highly active enzymes to remove ROS. These theoretical analyses suggest that the concentration of chemical antioxidants that would alter endogenous ROS cannot be reached.

With characteristic foresight, the authors used the *E. coli* cell to address any concerns that the in vitro experiments were not capturing the whole story. The calculations were based on wild-type cells that have an arsenal of ROS-scavenging enzymes. A critical next step was to address the effect of these same antioxidants on cells that were demonstrably undergoing oxidative stress. *E. coli* has two superoxide dismutase enzymes encoded by *sodA* and *sodB*, and *sodAB* mutant strains are compromised in their ability to quench endogenous superoxide anions (Carlioz & Touati, 1986). Korshunov and Imlay used *sodAB* mutant strains to test whether antioxidants removed superoxide in vivo when provided exogenously at typically used concentrations. Antioxidants were tested for the ability to reverse the consequences of accumulated superoxide anions in a *sodAB* mutant strain in three ways. First, under aerated conditions strains lacking SodA and SodB do not grow due to superoxide-mediated damage to critical [4Fe-4S] dehydratases (Kuo & Rose, 1987). Addition of antioxidants thiourea, ascorbate, glutathione, or N-acetylcysteine failed to improve growth of a *sodAB* strain under aerated conditions. This result was particularly striking since as little as 10% of the normal levels of SOD allows full growth of the *sodAB* mutant in aerated conditions (Gort & Imlay, 1998). In other words, at the commonly used concentrations, antioxidants failed to restore even 10% of the activity encoded in a wild-type cell. Second, as a mononuclear Fe^{2+} enzyme, threonine dehydrogenase is a target of superoxide anions. Predictably, threonine dehydrogenase activity is reduced in a *sodAB* strain (Anjem & Imlay, 2012). Again, when a *sodAB* strain was grown in the presence of chemical antioxidants the activity of threonine dehydrogenase did not increase. Finally, a *sodA* strain is more sensitive to the redox-cycling antibiotic paraquat than

the wild-type strain due to the superoxide anion produced. As might be predicted from the results above, the addition of antioxidants had no beneficial effect on growth of the *sodA* strain in the presence of paraquat. Thus, the data from growth and biochemical experiments that monitor in vivo effects supported the conclusions reached using calculations that incorporated in vitro data. While the conclusion that the effect of antioxidants is not due to quenching oxidants may seem counterintuitive, the data presented by Korshunov and Imlay are unequivocal, and have significant implications for investigators using these compounds as supplements (Korshunov & Imlay, 2024).

Thus, the study by Korshunov and Imlay convincingly shows that the ability of antioxidants to ameliorate the effects of various treatments that cause cell death is not an indication of underlying ROS stress. Some of the experiments that supported this conclusion were highlighted above. To go beyond merely dismissing the frequent explanation for the impact of antioxidants, the authors probed the basis of the suppression by antioxidants observed with many stresses. Two points came together in generating the hypothesis that was pursued. First, the authors noted that at the concentrations provided to suppress stress, antioxidants (e.g., thiourea [Keren et al., 2013]) typically slow growth of a bacterial culture. Second, many if not most, agents are less efficient at killing bacteria when cell growth is slowed. Thus, *Korshunov and Imlay proposed that antioxidants mitigate the effect of diverse stresses by slowing growth*. Results from experiments involving norvaline support this hypothesis. Norvaline is a non-proteinogenic amino acid that slows growth by inhibiting protein synthesis (Reitz et al., 2018). Norfloxacin is a quinolone antibiotic that belongs to the class of agents (described above) whose lethality is reversed by thiourea or other scavengers. Strikingly, the addition of norvaline inhibited the toxicity of norfloxacin, linking suppression to growth rate reduction. It was also particularly telling that antioxidants (e.g., thiourea) also protected against norfloxacin toxicity under anoxic conditions, when ROS are not produced. Thus, beyond showing that antioxidants do not control cell killing by scavenging ROS, this study provides insights into the true mechanism of suppression by antioxidants. When provided in concentrations that reduce stress causing cell death, antioxidants slow growth, which allows the strain to better deal with the consequences of the stress and retain viability. These data completed the circle, taking an observation that was itself perplexing: that multiple diverse stresses would act via a single mechanism, breaking down the inconsistencies and ultimately defining a model for the role of antioxidants in relieving cell death resulting from diverse stresses that is rigorously supported by both in vitro and in vivo experiments.

In total, this important study from the Imlay laboratory expands our understanding of the interplay between oxidants and antioxidants inside the cell and re-emphasizes the exquisite suite of scavenging enzymes that are present and active in a bacterial cell. Although the native stress response system can be overrun, for instance by redox-cycling compounds that increase internal ROS formation, this appears to be rare and usually not lethal. As such, the widespread idea that antioxidants protect growth by quenching stress-induced ROS needs to be re-evaluated. Importantly, the authors do not suggest they have disproven a connection between ROS and all cell stresses. Rather, they emphasize that antioxidants are not a valid way to diagnose endogenous ROS accumulation. In fact, there are several studies that have shown various antibiotic treatments do increase ROS. For example, treatment with CO-releasing molecules (CORMs) causes cell death that has been partially attributed to a surge in the concentration of ROS (Tavares et al., 2012). And some bactericidal (but not bacteriostatic) antibiotics have been shown to induce formation of hydroxyl radicals (Kohanski et al., 2007). These latter two cases are distinguished by the extent of the data supporting their conclusions and emphasize the need for rigorous evaluation proposed by the authors of the study highlighted here (Korshunov & Imlay, 2024). Despite the specificity of this study to *E. coli*, the logic used by Korshunov and Imlay can likely be extrapolated to other bacteria. Beyond the context of ROS, the underlying message has broad implications. Korshunov and Imlay elegantly underlined the benefit of using an approach to physiology that is multi-pronged, utilizing in vitro, in vivo and theoretical approaches when formulating conclusions that reveal the striking malleability of metabolic networks.

AUTHOR CONTRIBUTIONS

Diana M. Downs: Conceptualization; writing – review and editing. **Robert K. Poole:** Conceptualization; writing – review and editing.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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