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Original Contribution

Real-time investigation of antibiotics-induced oxidative stress and superoxide release in bacteria using an electrochemical biosensor

Xiaobo Liu [a], Mouna Marrakchi [a,b,c], Michael Jahne [d], Shane Rogers [d], Silvana Andreescu [a,*]

[a] Department of Chemistry & Biomolecular Science, Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5810, USA
[b] University of Carthage, Laboratoire d'Ecologie et Technologie Microbienne (LETMi), Institut National des Sciences Appliquées et de Technologie (INSAT), 1080 Tunis, Tunisia
[c] Tunis El Manar University, Higher Institute of Applied Biological Science of Tunis (ISSBAT), 1006 Tunis, Tunisia
[d] Department of Civil and Environmental Engineering, Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5710, USA

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ABSTRACT

The involvement of oxidative stress in the mechanism of antibiotics-meditated cell death is unclear and subject to debate. The kinetic profile and a quantitative relationship between the release of reactive oxygen species (ROS), bacteria and antibiotic type remain elusive. Here we report direct measurements and analytical quantification of the release of superoxide radicals ($O_2^{\bullet -}$), a major contributor to ROS, in antibiotics-treated bacterial cultures using a cytochrome *c* electrochemical biosensor. The specificity of electrochemical measurements was established by the addition of superoxide dismutase (SOD) which decreased the $O_2^{\bullet -}$ signal. Measurements using a general ROS-specific fluorescence dye and colony forming units (CFU) assays were performed side-by-side to determine the total ROS and establish the relationship between ROS and the degree of lethality. Exposure of *Escherichia coli* and *Listeria monocytogenes* cultures to antibiotics increased the release of $O_2^{\bullet -}$ radicals in a dose-dependent manner, suggesting that the transmembrane generation of ROS may occur as part of the antibiotic action. The study provides a quantitative methodology and fundamental knowledge to further explore the role of oxidative stress in antibiotics-meditated bacterial death and to assess physiological changes associated with the complex metabolic events related to oxidative stress and bacterial resistance.

1. Introduction

The continuous emergence of antibiotic resistant bacteria is of great scientific and practical interest [1]. Exploring the mechanisms of antibiotics action against bacteria is essential to improve the understanding of how antibiotics affect bacterial metabolism and address the issue of multidrug resistance. Several potential mechanisms of action have been proposed including inhibition of cell wall assembly, suppression of protein synthesis and disruption of DNA replication and repair. More recent studies have suggested the involvement of oxidative stress, and the release of reactive oxygen species (ROS) in the activity of antibiotics in bacteria, regardless of their molecular targets [2–4]. However, whether oxidative stress is involved in the mechanism of bactericidal antibiotics is still unclear and subject to debate [5].

The ROS hypothesis indicates that the action of bactericidal antibiotics involves the bacterial tricarboxylic acid cycle, NADH depletion, destabilization of iron–sulfur clusters, and stimulation of the Fenton reaction with formation of hydroxyl radicals in both gram-negative and gram-positive bacteria [6]. Recent work using a panel of biochemical and biophysical assays has further supported this hypothesis and provided additional evidence that antibiotics induce significant redox alterations that contribute to cellular damage and death [2]. Antibiotics were found to induce dynamic changes in cellular respiration and lethal levels of intracellular H_2O_2 that could be diminished by addition of antioxidants, such as glutathione and ascorbic acid. Other works also identified the presence of ROS in antibiotic-treated bacteria for several quinolone antibiotics, by using the nitroblue tetrazolium reduction (NBT) assay [7]. Excessive release of ROS species, including H_2O_2, hydroxyl ($HO^{\bullet}$) and superoxide ($O_2^{\bullet -}$) radicals is harmful to cells and may cause severe damage, including necrosis and apoptosis [8]. Excessive ROS has been considered a sign of oxidative stress. The release of hydroxyl radicals in bacteria was evidenced with a hydroxyl radical specific dye, hydroxyphenyl fluorescein (HPF) [6,9]. The survival percentage of antibiotic-treated bacteria increased after adding thiourea, a hydroxyl radical scavenger, providing further evidence that the observed effect is due to oxidative stress [6,9,10]. Other studies reported a relation between antibiotic susceptibility and ROS release, detected by chemiluminescence

* Corresponding author.
E-mail address: eandrees@clarkson.edu (S. Andreescu).

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with lucigenin for $O_2^{\bullet-}$ and luminol for other ROS species [11,12]. An increase in ROS species, including $O_2^{\bullet-}$ was observed in chloramphenicol treated *Staphylococcus aureus* and *Escherichia coli* and in ciprofloxacin treated *S. aureus, E. coli and Enterococcus faecalis* [12]. Antibiotic-resistant strains showed reduced levels of $O_2^{\bullet-}$ production in ciprofloxacin treated *S. aureus* [11]. The involvement of $O_2^{\bullet-}$ and H_2O_2 was also demonstrated in ciprofloxacin treated *E. coli* [13]. These studies provide cumulative evidence that ROS contribute to antibiotic lethality [6,13].

While a number of studies have indicated the possible connection between cell death from antibiotics action and ROS production, several recent reports have questioned this connection [14–16]. These studies showed that ampicillin, norfloxacin and kanamycin do not increase H_2O_2 formation in *E. coli* [14], and reported that kill does not depend on the presence of oxygen, and is not related to ROS production [14–16]. However, new work by Collins and collaborators, using a more extensive battery of tests have reconfirmed the ROS hypothesis, showing that antibiotic lethality is accompanied by ROS generation, and that environmental variables play a role when performing such measurements [2]. Fang attributes the divergent results to differences in experimental protocols and the limitations of measurement techniques to assess oxidative stress and ROS species [5]. Such limitations include lack of specificity of fluorescence dyes [17,18] and/or the inhibitors for ROS species [14] and potential competitive redox processes involving the dye (e.g. fluorescence based). These studies indicate that better methodologies are needed to study the complex processes of antibiotic-induced responses and test the hypothesis that ROS is indeed involved in the kill of bacteria by antibiotics [14–16].

Methods commonly used to study ROS release in antibiotics-treated bacteria include: growth inhibition assays, cell viability tests (counts of colony forming units (CFU) on nutrient agars), chemiluminescence, colorimetric and fluorescence spectroscopy methods. Growth inhibition and viability tests are primarily used to determine the bacteriostatic and/or lethal effect of the antibiotics, but limited mechanistic information of the drugs against bacteria can be extracted from the results. Spectrophotometric, spectrofluorometric and chemiluminescence methods can provide evidence of the presence of oxidative stress but these methods involve addition of exogenous reagents that might react with other components in the bacteria culture and lack the specificity for individual ROS species. More direct measurements of the markers of oxidative stress would be a useful addition to the arsenal of tests used to study physiological aspects related to redox mechanisms in bacteria.

In this paper, we report dynamic release of $O_2^{\bullet-}$ anion radicals in antibiotic-exposed bacteria using an electrochemical biosensor that allows continuous measurement of $O_2^{\bullet-}$ directly in the culture, without addition of exogenous reagents. We use a miniaturized cytochrome *c* based biosensor developed previously [16]. The method consists of a gold wire electrode modified with cytochrome *c*, immobilized via a mixed thiol layer [19]. The principle of detection is based on the redox reaction of the immobilized cytochrome c with the released $O_2^{\bullet-}$ at the electrode surface [19]. The immobilized cytochrome *c* is reduced by $O_2^{\bullet-}$ and subsequently oxidized at the electrode, generating a redox current. As compared to other methods, the cytochrome *c* biosensor has several advantages including: (1) direct measurement in the bacterial culture with minimum perturbation of the environment, (2) reagentless operation, (3) high sensitivity and selectivity and (4) measurements are quantitative (with appropriate calibration in the bacterial growth medium). These sensors are inexpensive to produce and relatively easy to use [20]. Electrochemical cytochrome *c* based biosensors have been applied to quantify $O_2^{\bullet-}$ primarily in standards of enzymatically-produced $O_2^{\bullet-}$ [21]. To our knowledge such studies have not been conducted in bacterial cultures. Therefore, in this study, we first evaluated the sensitivity and specificity of measurements in the bacteria culture medium. The sensor was then used to detect $O_2^{\bullet-}$ radicals released from antibiotics-treated Gram-negative and Gram-positive bacteria (*E. coli* and *L. monocytogenes*, respectively). Ampicillin (a β-lactam antibiotic), norfloxacin (a quinolone), and kanamycin (an aminoglycoside), were used as model antibiotics possessing different molecular targets. Specificity of the biosensor measurements was established using SOD. The method allowed us to measure the continuous release of $O_2^{\bullet-}$ in bacterial cells and estimate the rate of $O_2^{\bullet-}$ production.

CFU assays were used to screen the killing effect of the antibiotics through a wide range of concentrations. Furthermore, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), a general fluorescent indicator for ROS, was also used alongside electrochemical measurements to determine the presence of ROS in the antibiotic-treated bacteria. Results from this work indicate that antibiotics trigger the release of $O_2^{\bullet-}$ in *E. coli* and *L. monocytogenes* in a dose-dependent manner. This study also provides a methodology for assessing the dynamics of $O_2^{\bullet-}$ release in bacteria cultures and demonstrates the potential of this approach for studying oxidative stress mechanisms in bacteria.

2. Experimental

2.1. Materials and reagents

XOD from bovine milk (EC1.17.3.2), cytochrome *c* from horse heart, superoxide dismutase (SOD), hypoxanthine (HX), 11-mercapto-1-undecanol (MU), 3-mercapto-1-propionic acid (MPA),1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma (St Louis, MO) and used as received. 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was obtained from Invitrogen, Life Technology (Carlsbad, CA). Ampicillin sodium salt and kanamycin sulfate were purchased from Amresco (Framingham, MA). Norfloxacin nicotinate was purchased from Enzo Life Science (Farmingdale, NY). Difco™ LB broth and agar, Miller (Luria–Bertani) and BBL™ Brain Heart Infusion broth and agar were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Deionized water from Direct-Qsystem (Millipore, Billerica, MA) with a resistivity of 18.2 MΩ cm was used to prepare all solutions.

2.2. Instrumentation

Gold wires with the diameter of 0.5 mm purchased from Goodfellow Corporation (Coraopolis, PA) were used to prepare the working electrodes. A Mini Shaker Incubator from VWR (Radnor, PA) was used for the bacterial growth. All electrochemical measurements were run on a CHI 1232b electrochemical analyzer (CH Instruments, Inc., Austin, TX). A Cary Eclipse Fluorescence Spectrometer (Agilent Technologies, Santa Clara, CA) was used for the fluorescence measurements.

2.3. Preparation and electrochemical characterization of antibiotic stock solutions

Ampicillin, norfloxacin and kanamycin were obtained in their salt form to facilitate the dissolution in aqueous medium. The concentration of antibiotics stock solution, preparation procedures and storage conditions followed the protocols described by Cockerill [22]. Since electrochemical measurements are used to assess