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New aspect of the synergistic antibacterial action of ampicillin and gentamicin

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Abstract

A new aspect in the action of ampicillin and gentamicin was detected in *Enterococcus faecalis*. Reactive oxygen species (ROS) increased in sensitive strains during treatment with each antibiotic up to a certain concentration of antibiotic, above which ROS diminished as a consequence of oxidative stress. Tiron, a scavenger of the superoxide anion O_2^-, counteracted the effect of the generated ROS. The oxidative stress was a consequence of an increase in ROS in the cytoplasm of bacteria, as observed by the nitroblue tetrazolium reaction. The viability of sensitive strains was significantly reduced at concentrations of antibiotics that increased the ROS, and this increment was parallel to the bactericidal effect. Sensitive *E. faecalis* strains showed an immediate increase of ATP in the presence of both antibiotics, thus an energy-dependent process had been triggered, indicating a bacterial reaction against the stress. The combination of both antibiotics augmented the effect of ROS, which helps to explain the synergism between ampicillin and gentamicin.

Keywords: *Enterococcus faecalis*; Ampicillin; Gentamicin; Oxidative stress; Antibiotic synergy; ATP level

1. Introduction

Resistance to antimicrobial agents continues to be a major problem in patients infected with *Enterococcus faecalis*, as antibiotic-resistant variants may arise regularly. Hospital infection controls attempt to maintain resistance at low levels, thus reducing antibiotic selective pressure; however, it is common to find resistance to β-lactamases and aminoglycosides in isolates obtained from clinical specimens [1–4].

The increasing prevalence of ampicillin and high-level gentamicin resistance compromises traditional therapy, and study of these resistant strains is necessary since the therapeutic possibilities for patients infected with these enterococci are limited [5]. One therapy reserved for treatment of these serious resistant infections is the use of synergistic combinations of a β-lactamase agent plus an aminoglycoside [6]. However, when an *E. faecalis* strain acquires aminoglycoside resistance mediated by aminoglycoside-modifying enzymes, the synergistic bactericidal effect is eliminated.

The combination of ampicillin with an aminoglycoside can be used only in patients infected with enterococci with low-level aminoglycoside resistance [7]. The synergy between ampicillin and gentamicin is a potential useful strategy in the treatment of gentamicin-resistant enterococci when the gentamicin maximum inhibitory concentration (MIC) is less than 500 μg/mL. The determination of gentamicin MICs is an in vitro predictor of synergy with ampicillin. Strains that show synergy between both antibiotics exhibit an increase in the killing effect of ampicillin alone; whilst strains with gentamicin MICs between 500 and 2000 μg/mL do not demonstrate synergy [8]. Nowadays, it is necessary to clarify the metabolic causes of synergy between ampicillin and gentamicin.

In this regard, antibiotics have not been adequately investigated as a possible cause of oxidative stress in bacteria. Little is known about the production of reactive oxygen species (ROS) in strains susceptible to antimicrobial agents, and although the production of oxidative stress was recently

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described in bacteria treated with ciprofloxacin, chloramphenicol, ceftazidime and piperacillin [9,10], more investigation is still necessary to determine whether oxidative metabolism can be affected by ampicillin and gentamicin in *E. faecalis*.

The aim of this study was to investigate the amount of ROS produced by *E. faecalis* in the presence of ampicillin or gentamicin alone, or ampicillin/gentamicin combined, and also to determine the alteration of ATP provoked as a consequence of oxidative stress in sensitive strains, with the aim of detecting metabolic alterations associated with the synergistic effect. This investigation of the metabolic changes provoked by ampicillin or gentamicin might clarify new aspects of their antibacterial mechanism.

2. Materials and methods

2.1. Bacterial strains

Enterococcus faecalis isolates from human infections were tested for their antibiotic sensitivity to ampicillin and gentamicin. The strains studied were isolated from different infections, including devices colonised by bacteria. *Enterococcus faecalis* ATCC 29212 was included in the investigation as control. Bacteria were cultured in trypticase soy agar (TSA) with 6% blood and were identified by different tests: catalase, bile esculin, hippurate, pyrrolidine assay, tellurite and carbohydrate fermentation.

2.2. MIC assay

MIC was determined using the standard tube dilution method in Müeller–Hinton medium. Strains from 24-h cultures in trypticase soy broth (TSB) were diluted in Müeller–Hinton medium to 10^6 colony-forming units (CFU)/mL and incubated at 37 °C for 10 min. Ampicillin (0.5–16 μg/mL) or gentamicin (0.5–800 μg/mL) was then added at various concentrations. Bacterial growth was observed after 24 h incubation, according to the National Committee for Clinical Laboratory Standards (NCCLS) indications [11].

2.3. Determination of ROS by chemiluminescence (CL) assay

Oxidative stress of *E. faecalis* was investigated in a Bio-Orbit luminometer after 5 s, 10, 15 and 30 min incubation with antibiotics (0.5–16 μg/mL ampicillin or 0.5–800 μg/mL gentamicin). The light emitted by ROS was expressed as relative light units (RLU) at different times in seconds after subtraction of the background. Bacteria were cultured in TSB at 37 °C for 24 h and their final density was adjusted to 10^8 CFU/mL by counting colonies. In addition, optical density at 600 nm (OD_{600}) was determined to achieve the same optical density in each assay. A volume of 0.1 mL of bacterial suspension with 10^6 CFU was incubated with 0.1 mL of 75 μg/mL lucigenin (bis-*N*-methylacridinium nitrate), 0.1 mL phosphate buffer (pH 7.2) and 0.1 mL ampicillin (0.5–16 μg/mL) or gentamicin (0.5–800 μg/mL), plus 0.1 mL dimethylsulphoxide (DMSO) triggered at the moment of RLU determination. Controls for production of ROS were performed with bacteria in the absence of antibiotics. Assays for synergism were performed with 0.1 mL of the combination of 8 μg/mL ampicillin plus 500 μg/mL gentamicin.

2.4. Assays with a scavenger of ROS

A volume of 0.1 mL of bacterial suspension with 10^6 CFU was incubated with 0.1 mL of 75 μg/mL lucigenin and 0.1 mL of 50 nM 4,5-dihydroxy-1,3-benzene disulphonic acid (Tiron), a scavenger of ROS and principally of superoxide anion (O_2^-), plus 0.1 mL of antibiotics in the same concentrations as used in the determination of ROS by CL assay, and finally 0.1 mL of DMSO was added.

2.5. Nitroblue tetrazolium reduction

A volume of 0.1 mL of bacterial suspension in TSB (OD_{600} 1.0) was incubated with 0.1 mL of 3 mg/mL ciprofloxacin and 0.5 mL of 1 mg/mL nitroblue tetrazolium (NBT) for 30 min at 37 °C. Then, 0.1 mL of 0.1 M HCl was added and the tubes were centrifuged at 1500 × *g* for 10 min. Absorbance of supernatants was measured at 575 nm (extracellular ROS). The separated pellets were treated with 0.6 mL DMSO to extract the reduced NBT; finally, 0.8 mL of TSB was added and absorbance was determined at 575 nm (intracellular ROS).

2.6. Relationship between bactericidal effect and oxidative stress

A gentamicin-sensitive and -resistant strain (both sensitive to ampicillin) were cultured for 24 h in TSB with 10 μg/mL of gentamicin and 8 μg/mL of ampicillin. Samples of these cultures were obtained at different times (2 s, 2 h, 4 h, 8 h and 24 h) to determine: (a) ROS production by CL without the addition of antibiotics when performing the assay; and (b) the viability by determining the number of bacteria (CFU/mL) seeding from 0.1 mL of cultures in TSA plates incubated for 24 h at 37 °C.

2.7. Luciferase–luciferin assay to determine ATP in bacteria

Enterococcus faecalis strains were cultured in TSB at 37 °C for 24 h and bacteria were then exposed to 8 and 16 μg/mL ampicillin, 500 and 800 μg/mL gentamicin, or a combination of both antibiotics at 37 °C for 30 min. ATP was extracted by heat treatment; 1 mL of bacterial culture (10^6 CFU/mL) was mixed with 3 mL of water at 100 °C and