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Bactericidal effect of colistin on planktonic *Pseudomonas aeruginosa* is independent of hydroxyl radical formation

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ABSTRACT

The bactericidal effect of several major types of antibiotics has recently been demonstrated to be dependent on the formation of toxic amounts of hydroxyl radicals (OH•) resulting from oxidative stress in metabolically active cells. Since killing by the antimicrobial peptide colistin does not require bacterial metabolic activity, we tested whether the bactericidal effect of colistin depends on the formation of OH•. In *Pseudomonas aeruginosa* cultures, OH•-mediated killing by ciprofloxacin was demonstrated by decreased bacterial survival and induction of 3′-(*p*-hydroxyphenyl) fluorescein (HPF) fluorescence. OH•-mediated killing by ciprofloxacin was further confirmed by rescue of cells and reduction of HPF fluorescence due to prevention of OH• accumulation by scavenging with thiourea, by chelating with dipyridyl, by decreasing metabolism as well as by anoxic growth. In contrast, no formation of OH• was seen in *P. aeruginosa* during killing by colistin, and prevention of OH• accumulation could not rescue *P. aeruginosa* from killing by colistin. These results therefore demonstrate that the bactericidal activity of colistin on *P. aeruginosa* is not dependent on oxidative stress. In conclusion, antimicrobial peptides that do not rely on OH• formation should be considered for treatment of Gram-negative bacteria growing at low oxygen tension such as in endobronchial mucus and paranasal sinuses in cystic fibrosis patients, in abscesses and in infectious biofilm.

1. Introduction

Polymyxin E (colistin) is a bactericidal lipopeptide antibiotic active against Gram-negative bacteria. The mechanisms of action of the cationic colistin include disruption of the cytoplasmic membrane through interaction with cationic binding sites on the cell surface lipopolysaccharides, which destabilises the outer membrane and promotes its own uptake [1]. However, solid data to support disruption of the cytoplasmic membrane of Gram-negative bacteria by polymyxins at clinically relevant concentrations remain to be established. Owing to its nephrotoxicity, use of colistin in patients with cystic fibrosis (CF) has been restricted to inhalation treatment of lung infections caused by *Pseudomonas aeruginosa*. However, colistin has resurfaced as a last-line treatment option for multidrug-resistant organisms such as *P. aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* as well as for carbapenemase-producing Enterobacteriaceae in other infections [2].

Recently, a common mechanism of killing by bactericidal antibiotics has been suggested to involve the formation of harmful hydroxyl radicals (OH•) through the Fenton reaction. According to this model, transient hyperoxidation by the respiratory chain of NADH from the tricarboxylic acid (TCA) cycle leads to production of superoxide (O_2^-) causing release of iron from iron–sulphur clusters resulting in substrates for generation of toxic amounts of OH• by the Fenton reaction. Consequently, it is suggested that killing by bactericidal antibiotics depends on the bacterial metabolic state, i.e. the TCA cycle [3]. According to the proposed underlying mechanism, both O_2^- and H_2O_2 formation are involved in the production of OH [3]. But while O_2^- and H_2O_2 can be enzymatically eradicated by superoxide dismutases, catalases and peroxidases, no known enzyme is able to catalyse the cellular detoxification of OH, which may induce lethal oxidative lesions on proteins, lipids and DNA [4]. Killing by colistin, however, is reduced by bacterial

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metabolic activity [5,6], suggesting that the bactericidal activity of colistin does not necessitate formation of OH• by hyperoxidation of NADH from the TCA cycle. In addition, it may be presumed that formation of OH• requires the presence of molecular oxygen (O_2), which emphasises the need to consider the type and location of infection carefully when selecting optimal antibiotic treatment. In particular, the infected mucus of CF patients contains anaerobic zones [7], which are mainly due to oxygen consumption by the summoned neutrophils [8]. Furthermore, decreased mucosal O_2 tension prevails in the paranasal sinuses of CF patients, which represent a gateway for infection in the lung [9]. Based on this knowledge, we were interested in investigating: (i) the role of reactive oxygen species (ROS) formation during killing of *P. aeruginosa* by colistin; (ii) the effect on bacterial killing of ROS modulators such as thiourea for scavenging of OH• [10] and chelation of ferrous iron with dipyridyl in order to prevent accumulation of OH• through Fenton reactions [11]; and (iii) the effect of limitation of nutrients and O_2 on OH• formation and bacterial killing during antibiotic treatment. Therefore, we have constructed bacterial killing curves and estimated OH• formation during antibiotic treatment in aerobic and anaerobic conditions as well as during nutrient limitation. For comparison, ciprofloxacin was used as an antipseudomonal drug, with the effect depending on oxidative stress in metabolically active cells [3].

2. Materials and methods

2.1. Bacterial strain, media and antibiotics

Wild-type *P. aeruginosa* strain PAO1 used for the experiments was obtained from the *Pseudomonas* Genetic Stock Centre (http://www.pseudomonas.med.ecu.edu; strain PAO0001). Three clinical *P. aeruginosa* strains isolated from CF patients with chronic lung infection were also tested for their response to colistin: 9B, a non-mucoid isolate; 9A, a mucoid isolate; and 4137, a resistant mucoid isolate. Strains were grown in Luria–Bertani (LB) broth [5 g/L yeast extract (Oxoid, Roskilde, Denmark), 10 g/L tryptone (Oxoid) and 10 g/L NaCl (Merck, Rahway, NJ), pH 7.5], incubated overnight at 37 °C and shaken at 170 rpm. The bactericidal antibiotics colistin sulphate salt (Sigma–Aldrich, Brøndby, Denmark) and ciprofloxacin (Bayer, Leverkusen, Germany) were used.

For the determination of PAO1 CFU counts, solid lactose agar plates [10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, 11 g/L agar, 0.05 g/L detergent, 1 g/L $Na_2S_2O_3{\cdot}5H_2O$, 0.1 g/L bromothymol blue, 9 g/L lactose, 0.4 g/L glucose, pH 8.0 (State Serum Institute, Copenhagen, Denmark)] were used to select for Gram-negative bacteria. All plates were incubated overnight at 37 °C.

2.2. Susceptibility testing

To avoid the limited accuracy caused by the poor agar diffusion characteristics of colistin, the minimum inhibitory concentration (MIC) was determined by Etest (AB BIODISK, Solna, Sweden) as recommended [12] following the instructions of the manufacturer. Anaerobic Etests were performed on LB agar plates with 10 mM KNO_3 equilibrated in an anaerobic bench (Concept 400; Ruskinn Technology, Pencoed, UK) for 3 days to remove O_2.

Survival curves and OH• formation were assayed to investigate the effect of O_2 and nutrient limitation on *P. aeruginosa* treated with colistin, ciprofloxacin or hydrogen peroxide. Inocula of exponentially growing cells were prepared from overnight cultures diluted in LB broth to an optical density at 600 nm (OD_{600}) of 0.1 and re-grown to OD_{600} = 0.4 (ca. 10^8 cells/mL). To achieve ca. 10^6 cells/mL, the re-grown cultures were diluted 100-fold in normoxic LB, oxygen-depleted LB broth or 0.9% saline. To detect OH• formation, 5 μM 3′-(*p*-hydroxyphenyl) fluorescein (HPF) (Molecular Probes, Eugene, OR) was added. Antibiotic treatment was carried out by addition of 1.5 mg/L colistin and 0.25 mg/L ciprofloxacin. Thiourea (150 mM) (Sigma) was added for scavenging of OH•, and the iron chelator 2,2′-dipyridyl (4 mM) (Sigma) was added to inhibit the Fenton reaction. To ensure metabolic activity during the study of the effect of oxygen, all media were supplemented with $NaNO_3$ (10 mM) (Sigma) to allow anaerobic respiration. All oxygen-depleted cultures were prepared in an anaerobic bench using equilibrated media with a partial pressure of <0.02% O_2 as determined with a Multi-parameter Metre HQ40d (HACH Company, Loveland, CO).

To inhibit metabolism, re-grown overnight cultures were depleted of nutrients by 100-fold dilution in 0.9% saline. Antibiotics and HPF were added as described in the study of the effect of oxygen. Cultures were aliquoted in 1 mL glass vials (Schuettbiotec GmbH, Göttingen, Germany) and the anaerobic cultures were sealed with airtight lids. All cultures were covered by tin foil, incubated at 37 °C and shaken at 250 rpm. CFU/mL were estimated by plating of serial dilutions. In this assay, the lower level of detection for bacterial counts was 1 colony/plate, corresponding to 10 CFU/mL. Consequently, all samples with ≤1 colony/plate were recorded as 10 CFU/mL. OH• formation was monitored by flow cytometry for 3 h after addition of antibiotics. Every hour, samples were harvested and the airtight lids of the glass vials were opened to perform flow cytometric analysis and estimation of CFU/mL.

To detect simultaneous cell death and OH• formation during early colistin treatment, 1 mL of exponentially growing PAO1 (10^6/mL) in LB broth with 5 μM propidium iodide (PI) (P-4170; Sigma), 5 μM HPF and colistin (0, 0.125, 0.5, 2 and 8 mg/L) was added to a FALCON tube (352052; Becton Dickinson, Bedford, MA) together with phosphate-buffered saline, thiourea or dipyridyl. The tubes were incubated at 37 °C and were shaken at 250 rpm for 7.5, 15 and 30 min before estimating cell death by PI fluorescence following membrane disintegration as well as OH• induction by HPF fluorescence using flow cytometry. The early effect of colistin treatment (1.5 mg/L) in three clinical *P. aeruginosa* isolates from CF patients with chronic lung infections and PAO1 was also compared by flow cytometry.

2.3. Flow cytometry

Detection of OH• formation was performed using a FACSCanto™ flow cytometer (BD Biosciences, San Jose, CA) with a 488 nm argon laser and a 530/30 nm band pass emission filter for recording of HPF fluorescence in FL-1. Since a substantial increase in FL-1 fluorescence during colistin treatment without HPF was found, HPF fluorescence induced by the antibiotic treatment was expressed as the relative fluorescence intensity (RFI). To calculate the RFI for a particular colistin treatment, the FL-1 fluorescence induced by colistin treatment without HPF was subtracted from the FL-1 fluorescence induced by colistin treatment with HPF. PI fluorescence was collected through a 585/42 nm band pass emission filter and was recorded in FL-3. To maximise resolution, samples were analysed at low flow rate corresponding to 10 μL/min. At least 10,000 events were recorded for each sample. The following photomultiplier tube voltage settings were used for collection of linear amplified signals: 200 (FCS), 420 (SSC), 550 (FL-1) and 600 (FL-3). Cytometer Setup and Tracking Beads (BD Biosciences) were used for instrument calibration, and flow data were processed and analysed by Diva (BD Biosciences) and FlowJo v.7.6.5 (Tree Star Inc., Ashland, OR).

2.4. Statistics

Results from three individual experimental set-ups were evaluated by analysis of variance (ANOVA) using Bonferroni multiple