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Evaluation of electron spin resonance for studies of superoxide anion production by human neutrophils interacting with *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Abstract

The present study evaluates electron spin resonance (ESR) and the spin trapper 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) for analysis of superoxide radical production by human neutrophils interacting with viable *Staphylococcus aureus* and *Staphylococcus epidermidis* bacteria. To avoid auto-activation due to interaction with glass surfaces, neutrophils were preincubated in plastic tubes until the peak response was reached, and then transferred to a quartz flat cell to record the ESR spectra. The time point for peak response was identified by parallel analysis of the bacteria–neutrophil interaction using luminol amplified chemiluminescence. We found detectable ESR spectra from neutrophils interacting with as few as five bacteria of the weak activating *S. epidermidis* per neutrophil. Addition of the NADPH oxidase inhibitor diphenylene iodonium totally abolished spectra. Catalase, DMSO or an iron chelator had no impact on the produced spectra and ionomycin, a selective activator of intracellular NADPH oxidase, gave significant ESR spectra. Taken together, our results indicate that DEPMPO is cell permeable and detects NADPH oxidase derived superoxide anions formed in phagosomes or released by human neutrophils phagocytosing viable *S. aureus* and *S. epidermidis*. The technique may be used as a sensitive tool to evaluate superoxide anion production in human neutrophils.

Keywords: Phagocytosis; DEPMPO; Spin trapping; Staphylococci

1. Introduction

Free radicals, and other reactive oxygen species (ROS), are formed by professional phagocytes (granulocytes, monocytes and macrophages) in the first line of defence against intruding microorganisms. The phagocytes, armed with a multicomponent enzyme, the NADPH oxidase, generate superoxide ($O_2^{\cdot-}$) by electron transfer from the cytosolic donor NADPH to molecular oxygen localized on the opposite side of the membrane, i.e. in the phagosome or extracellularly [1,2]. In the body, neutrophils are activated upon interaction with e.g. i) infecting microorganisms [3], ii) inflammatory mediators, iii) immune complexes in joint inflammation [4] and iv) biomaterials [5]. However, isolated neutrophils *in vitro* might become auto-activated by handling during isolation and incubation. Especially glass surfaces has been shown to cause auto-activation, which could affect the analysis [6].

Oxygen centred free radicals are highly reactive and short lived, which make them hard to study in biological systems. Therefore, most of the techniques currently used to analyze the products generated and/or released by phagocytes rely on indirect measurements using colorimetry, fluorometry and chemiluminescence methodology (reviewed in [7]). To

Abbreviations: CL, chemiluminescence; ESR, electron spin resonance; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMSO, dimethylsulfoxide; DPI, diphenylene iodonium; DTPA, diethylenetriaminepentaacetic acid; HRP, horseradish peroxidase; KRG buffer, Krebs–Ringer glucose buffer; ROS, reactive oxygen species; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; SOD, superoxide dismutase.

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distinguish between intracellular production and extracellular release of ROS, luminol-amplified chemiluminescence (CL) in the presence of SOD and catalase is a well documented and widely spread method [8–10]. Luminol is membrane permeable and thus excited by both intra- and extracellularly produced ROS [11]. The specificity of luminol dependent CL, regarding the molecular mechanism responsible for excitation of the dye has, however, been debated. Further studies using alternative methods to analyze oxygen metabolites generated by professional phagocytes and to distinguish between intracellular production and extracellular release of phagocyte derived ROS are demanded.

ESR (electron spin resonance) is the only employed technique for *direct* detection and identification of free radicals. Although the sensitivity can be as high as 10^{-10} M, the method is not sufficient to detect short-lived species such as $O_2^{\cdot-}$ and $HO^{\cdot}$, unless the measurement is performed at low temperatures. Therefore, in biological samples, spin trapping is used to create long-lived radicals. The spin traps 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) [12], and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) [13,14] are widely used for detection of several radicals including superoxide anion radical, hydroxyl radical and various sulfur- and carbon centred organic radicals. For detection of superoxide radicals, DEPMPO has been preferred due to higher sensitivity and stability. The ESR technique has so far been utilized for qualitative and semi quantitative assessment of oxidative burst in phagocytes activated by phorbol myristate acetate (PMA), the bacteria derived peptide formyl-methionyl-leucyl-phenylalanine (fMLP), and lipopolysaccharide [15], as well as particulate prey such as yeast/zymosan [16–19]. Although all these studies document detectable DMPO derived ESR spectra after activation of neutrophils, there is a lack in consensus concerning location of the detected spin adducts. Moreover, investigations of phagocyte inflammatory response induced by bacteria using DEPMPO are lacking.

The aim of this study was to establish an ESR-based method, using DEPMPO as a spin trap, to follow ROS production from neutrophils (the most abundant of the granulocytes) during interaction with *S. aureus* and *S. epidermidis* bacteria with special focus on optimizing the detection of ROS production and diminishing neutrophil auto-activation. Furthermore, we assessed if the method could be used to distinguish intracellular ROS production from extracellular release of ROS.

2. Materials and methods

2.1. Isolation of neutrophils

Human granulocytes were isolated from heparinized venous blood as described earlier [20] with some modifications [10]. The blood was centrifuged on a gradient of Polymorphprep® and Lymphoprep® (Axis-Shield PoC AS, Oslo, Norway), and the granulocyte layer was collected and washed. Erythrocytes were removed by twofold hypotonic lysis, and after an additional washing, granulocytes were resuspended in Krebs–Ringer glucose (KRG) buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 10 mM glucose, pH 7.3) supplemented with 1 mM $CaCl_2$. The viability of the cells was >98%, as assessed by trypan blue exclusion. Most of the granulocytes (98%) were neutrophils, based on morphological studies, and therefore they are henceforth named neutrophils. The number of neutrophils was adjusted to 10^7/ml, and the cells were stored on melting ice and used within 4 h.

2.2. Cultivation and preparation of *S. epidermidis* and *S. aureus*

A clinical isolate of *S. epidermidis* from an infected hip prosthesis [21] was stored at −70 °C. Cultures were grown on blood agar plates for 24 h at 37 °C, after which 2–3 colonies were transferred to Mueller-Hinton broth, and the bacteria were cultivated for another 18–20 h at 37 °C under agitation. *S. aureus* Wood 46, stored at −70 °C, was grown over night in Mueller-Hinton broth. A small volume of this culture was inoculated into fresh broth and the bacteria were grown for an additional 2 h. This yielded bacteria in the exponential growth phase. Both bacteria strains were washed once, opsonized in 25% normal human serum for 20 min, washed twice, resuspended in KRG, and diluted to 10^8 colony-forming units/ml by measuring the optical density.

2.3. Chemiluminescence

A six-channel chemiluminometer Biolumat LB 9505C (Berthold Co, Wildbaden, Germany) was used. Neutrophils (10^6 cells) were suspended in KRG containing 56 μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma St Louis, MA, USA), and 8 U horseradish peroxidase (HRP; Roche, Mannheim, Germany) in a total volume of 0.9 ml, and generated ROS were followed by continuous monitoring of the light production. To indirectly estimate the amount of ROS released from the neutrophils, 50 U of superoxide dismutase (SOD; Roche) and 2000 U of catalase (Roche) were added to the samples [9]. The samples were preincubated for 5 min in 37 °C, to yield a stable baseline, before adding the bacteria (in 0.1 ml KRG).

2.4. Electron spin resonance measurements

ESR spectra were obtained with a JES-FR30EX (Jeol, Tokyo, Japan) operating at X-band, modulation frequency 100 kHz, microwave power 4 mW, modulation amplitude 0.2 mT and time constant 1 s. Each ESR-sample contained 40 mM DMPO (Sigma) or 20 mM DEPMPO (Calbiochem, Ontario, Canada), 10^6 neutrophils, 10^7 bacteria, in a total volume of 500 μl KRG and the samples were preincubated in Eppendorf tubes in a water bath at 37 °C for 25 and 45 min for *S. aureus* and *S. epidermidis*, respectively. The samples were then loaded into an ES-LC-12 quartz flat cell, which was thermostated at 37 °C by circulating water while recording the ESR spectra. In selected experiments the reaction mixture was supplemented with SOD (50 U/ml), catalase (2000 U/ml), dimethylsulfoxide (DMSO, 1% [v/v]), diethylenetriaminepentaacetic acid (DTPA, 0.1 mM) [22],