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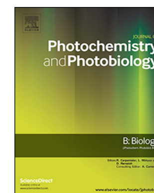

Porphyrins and flavins as endogenous acceptors of optical radiation of blue spectral region determining photoinactivation of microbial cells

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A B S T R A C T

It is shown that exposure of suspensions of gram-positive *Staphylococcus aureus*, gram-negative *Escherichia coli* and yeast-like fungi *Candida albicans* to laser radiation of blue spectral region with 405 and 445 nm causes their growth inhibition without prior addition of exogenous photosensitizers. It is experimentally confirmed that compounds of flavin type capable of sensitizing the formation of reactive oxygen species can act as acceptors of optical radiation of blue spectral region determining its antimicrobial effect along with endogenous metal-free porphyrins (the role of endogenous porphyrins has been confirmed earlier by a number of researchers).

The participation of these compounds in the antimicrobial effect of laser radiation is supported by the registration of porphyrin and flavin fluorescence in extracts of microbial cells upon excitation by radiation used to inactivate the pathogens. In addition, the intensity of the porphyrin fluorescence in extracts of microbial cells in the transition from radiation with $\lambda = 405$ nm to radiation with $\lambda = 445$ nm decreases by 15–30 times, whereas the photosensitivity of the cells under study in this transition decreases only 3.7–6.2 times. The contribution of porphyrin photosensitizers is most pronounced upon exposure to radiation with $\lambda = 405$ nm (absorption maximum of the Soret band of porphyrins), and flavins – upon exposure to radiation with $\lambda = 445$ nm (maximum in the flavin absorption spectrum and minimum in the absorption spectrum of porphyrins). The ratio between the intensity of the porphyrin and flavin components in the fluorescence spectrum of extracts depends on the type of microbial cells.

1. Introduction

At the present time, the ability of optical radiation of blue spectral region to exert a bactericidal action on microbial cells *in vitro* is practically not questioned [1–23]. The damaging effect of optical radiation in the spectral range of 400–470 nm without exogenous dyes-photosensitizers was demonstrated in ≈ 40 types of bacteria, fungi, yeasts in > 130 publications [1–23 and references in the mentioned papers]. It is believed that the most effective radiation for photoinactivation of microbes in the visible spectral region is radiation in the region of 405–410 nm [24–27]. Upon exposure of cells to radiation with wavelength of about 450 and 470 nm, higher energy doses of radiation are required to detect a pronounced bactericidal effect [1,4,17,18,22]. In a number of studies it is demonstrated that gram-negative microbial cells are more resistant to the exposure to radiation of blue spectral region than gram-positive cells [11,13,15,16,19]. At the same time, the author [1] on the basis of the analysis of the regularities of photoinactivation of various types of microbial cells concluded that there is no basis for such an assertion.

Photodamage of microorganisms is caused by both monochromatic laser radiation [8,28–30] and quasi-monochromatic radiation of light-emitting diodes [2-7, 9-14, 16, 17, 19, 27, 28, 30], superluminescent diodes [18,21,22], narrow-band light emission from lamps isolated with monochromators or optical filters [23–27] as well as by broadband light of lamp sources in the blue spectral region [17,24]. At the same radiation intensity, photoinactivation effects are practically equivalent when using laser and LED sources with a wavelength of 405 nm [8,28,30,31] as well as narrow-band light emission from lamps in the same spectral range [27]. The photodestruction of microbial cells increases with an increase in the energy dose and, according to the data of [27,31], is practically independent of the ratio of the power density and the time at which this dose was collected. However, according to [29], under conditions of the same energy dose, intense radiation with a wavelength of 405 nm exerts a more pronounced bactericidal effect

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which is achieved with a short exposure time in comparison with the action of radiation with lower power density and a longer irradiation time. It is believed that the bactericidal effect is oxygen-dependent: the magnitude of photoinactivation effect decreases with a decrease in the oxygen concentration [11,32–35] and also with the addition of the acceptors and quenchers of reactive oxygen species to the irradiated suspension of cells [11,27,32,35,36]. Direct confirmation (using the method of electron paramagnetic resonance) of the possibility of generation of hydroxyl radicals and superoxide radical by microbial cells under the exposure to optical radiation of blue spectral region [17,20,37,38] and also singlet oxygen detected by its luminescence in the 1270 nm region upon the excitation of lysed periodontal pathogens *Aggregatibacter actinomycetemcomitans* [39] or porphyrin molecules extracted from *Propionibacterium acnes* [40] was obtained. All this allows us to conclude that bactericidal effect upon exposure to the optical radiation of blue spectral region has a photochemical but not a photothermal nature. Indeed, when the temperature of the irradiated cell suspension was controlled using the thermal imaging technology or thermocouples, it was found that the temperature increase due to irradiation does not exceed 0.5–4.0 °C [6,33–35,37,38] and the medium temperature is much lower than the temperature that causes the death of microbial cells. Nevertheless, under certain conditions (especially upon exposure of cells attached to colored agar to intense radiation), the contribution of the photothermal component can be noticeable [41,42]. It is believed that the antibacterial effect of radiation of blue spectral region is primarily due to a disruption of the integrity and loss of the most important functional characteristics of cell membranes as well as the oxidation of intracellular DNA [7,43–46].

Based on the results of study of regularities of bactericidal effect of radiation of the blue spectral region on different types of microbial cells a number of authors concluded that observable effects are due to the sensitizing action of endogenous porphyrins [6,11,15,20,30,35,36,39,46–57] capable of generating reactive oxygen species. The presence of molecules of porphyrin photosensitizers in microbial cells is supported by the detection of characteristic porphyrin luminescence in extracts of various types of microbial cells with the most intense maximum in the region of 607–623 nm [6,11,20,30,39,48,49,52], 635 nm [30], 683 nm [47] or two maxima in the region of 620–630 and 667–690 nm [54–57]. Using high-performance liquid chromatography, the presence of the following metal-free tetrapyrroles in microorganisms was found: coproporphyrin, protoporphyrin, uroporphyrin [6,50–52,58,59] capable of absorbing light to generate reactive oxygen species. At the same time, it has been shown [6,11,33,45,50] that for many types of microbial cells there is no correlation between the concentration of endogenous porphyrins and the magnitude of bactericidal effect. It is suggested that the porphyrins contained in microorganisms are not the only factor determining their photosensitivity; in other words, in addition to porphyrin, other endogenous chromophores can contribute to the inactivation of pathogens when exposed to visible light. The assumption [39,44,54] of the possible role of endogenous flavin compounds to act as optical acceptors involved in sensitized damage to microbial cells has not yet been reliably confirmed experimentally.

The aim of the present study is to experimentally confirm that endogenous porphyrins and flavins can act as acceptors of optical radiation capable of causing the light-initiated death of diverse pathogens upon their exposure to laser radiation with $\lambda = 405$ and 445 nm. The study also aims to characterize the aforementioned chromophores using spectral-luminescent methods.

2. Materials and Methods

2.1. Microorganisms

The microorganisms used in the study were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231, all obtained from the American Type Culture Collection. Microorganisms were cultivated in tryptone soya agar (TSA) (*S. aureus, E. coli)* and sabouraud dextrose agar (*C. albicans*) (all Merck, Germany) at 37 °C during 24 h. All the experimental procedures were performed inside a sterile laminar flow hood.

2.2. Light Exposure of Bacterial Suspensions

Bacterial suspensions were prepared in sterile saline solution (0.9% NaCl) by serial dilutions using McFarland turbidity standard. A 3 ml of this suspension was transferred to the sterile cylindrical glass tubes with interior diameter of 1.4 mm and then the tubes were irradiated. The height of bacterial suspensions was 2.4 cm. The light sources used were semiconductor lasers generating in CW mode at wavelengths of 405 nm and 445 nm with smooth adjustment of power in the range of 50–500 mW (the development of the Institute of physics of NASB [60]). In order to irradiate whole volume of bacterial suspension the laser beam was expanded by a 10× telescope. The lasers were positioned directly above the tubes with samples. The irradiation time was varied in the range of 0–180 min. The power density of radiation in all experiments was kept constant – $P = (50 \pm 3)$ mW/cm^2. To control power density a power meter PM100D with photodiode sensor head S121C (Thorlabs GmbH, Germany) was used throughout of the study.

2.3. Assessment of Photoinactivation Effect

After irradiation, 100 µl of test and control samples were immediately plated onto agar plates using standard microbiological plating methods and incubated at 37 °C for 24 h before enumeration. The assessment of the viability of bacteria in test and control samples was carried out by counting the number of colony forming units (CFU) on plate. Photoinactivation effect (γ) was evaluated by the following equation

$$\gamma = (N_t/N_0) \times 100\% \tag{1}$$

where N_t – the number of CFU in test sample, N_0 – the number of CFU in control sample.

2.4. Studies of Spectral Characteristics of Bacterial Cells Extracts

The grown strains of microbial cells were washed three times by centrifugation at 5000*g*, for 5 min. The cell pellet, concentrated at the bottom of a centrifuge tube, was used to study the spectral characteristics of chromophores of porphyrin and flavin types. Identification of these chromophores was carried out using an ethyl acetate/acetic acid (3:1 v/v) mixture or a surface active non-ionic detergent Triton X-100 (Sigma-Aldrich, Inc.) as an extractant, which was added to the washed cells. Extraction was carried out by stirring using a Dragon Lab MX-S Vortex Mixer (Dragon Laboratory Instruments Ltd., China): for ethyl acetate/acetic acid mixture - for 5 h, for Triton X-100 - for 2 h followed by settling for 24 h. After centrifugation (5000 rpm, 10 min) the extracts were separated. The extract in Triton X-100 and part of the ethyl acetate layer was used directly for spectral-luminescent studies. The second portion of the ethyl acetate layer was washed twice with ice-cold distilled water. After separation of the mixture, the ethyl acetate layer was separated and mixed with an equal volume of 3 M HCl, shaken gently. After separation of the mixture into two layers, the hydrochloric acid fraction (bottom layer) was separated and the remaining ethyl acetate was re-extracted with an equal volume of 3 M HCl. This method was used to transfer the metal-free porphyrins, which can act as photosensitizers, from ethyl acetate to hydrochloric acid, without extracting, according to [50], heme-containing porphyrins.

The obtained fluorescence and fluorescence excitation spectra of the extracts were compared with the corresponding spectra of chemically pure compounds: coproporphyrin IX, protoporphyrin III, uroporphyrin III, and flavin mononucleotide phosphate from Sigma-Aldrich, Inc.

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