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Monitoring of hydrogen peroxide production under photodynamic treatment using protein sensor HyPer

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ABSTRACT

An interest to H_2O_2 accumulation under photodynamic treatment can be explained by its participation in intracellular signal cascades. It is important not only to detect H_2O_2 generation, but also to trace the dynamics of its intracellular content. In the present study the dynamics of cellular H_2O_2 content under photodynamic treatment was analyzed using genetically encoded reversible H_2O_2-sensitive sensor HyPer. Real-time detecting of H_2O_2 production after photodynamic treatment was performed using the protein sensor and individual features of action of different photosensitizers were revealed.

Photodynamic treatment with a number of chlorin and phthalocyanine photosensitizers was found to induce secondary production of H_2O_2 in the cells. Three types of dynamic responses were registered: monotonous increase of H_2O_2 level during the entire observation time in the presence of Fotoditazin and Holosens; transient short-term accumulation in the presence of Radachlorin and Phthalosens; and relatively low-level stable increase in the presence of Photosens. The listed photosensitizers differ significantly in intracellular localization and physicochemical properties, which can determine the differences in the response of H_2O_2 after the photodynamic treatment. In general, it has been shown that the rapid transient H_2O_2 response is typical for hydrophobic compounds localized in membrane cell structures, whereas in the presence of more hydrophilic dyes a prolonged monotonous H_2O_2 accumulation occurs.

1. Introduction

Photodynamic reactions include oxidation-reduction processes that occur with the participation of three components – the photoactive dye (photosensitizer), light and oxygen [1]. Under light irradiation the photosensitizer molecule passes into an excited state and transfers its energy to oxygen, resulting in the production of reactive oxygen species (ROS). Photosensitizers are widely used is photodynamic therapy (PDT) of cancer as well as a number of autoimmune and infectious diseases. The photosensitizers used at present are mostly agents based on hematoporphyrin, chlorins and bacteriochlorins, porphyrazines (phthalocyanines) [2,3]. Also 5-aminolevulinic acid is applied as the precursor of endogenously synthesized protoporphyrin IX. Preliminary administered photoactive dyes accumulate in the tumor; local irradiation causes the production of ROS and ultimately leads to death of tumor cells. Despite the fact that PDT is actively developing and the field of its application is constantly expanding, the molecular mechanisms underlying the photodynamic reaction have not yet been fully studied. At the same time, knowledge of these mechanisms is necessary for the rational development of new efficient photosensitizers as well as modifying their action and reducing side effects.

Until recently, singlet oxygen has been considered to be the main ROS generated under photodynamic treatment. However, an increasing amount of data indicates a significant contribution of other ROS, primarily hydrogen peroxide [4–7]. A particular interest to the study of the H_2O_2 accumulation under photodynamic treatment is due to its participation in intracellular signaling pathways [1,8]. It has been shown that the activity of NRF2, NF-κB and AP-1 transcription factors is modulated by ROS directly or through de novo synthesis [9–11]. Moreover, ROS produced during PDT can contribute to activation of cell death pathways through the regulation of Bcl-2 family proteins and caspases [12,13].

Abbreviations: cpYFP, circular permutated yellow fluorescent protein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMEM, Dulbecco's Modified Eagle's Medium; EPR, endoplasmic reticulum; LD50, half-lethal light dose; LED, light emitting diode; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PDT, photodynamic therapy; ROI, region of interest; ROS, reactive oxygen species

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Study of hydrogen peroxide production under photodynamic treatment can help to clarify the ways of PDT-induced cell death and find approaches to its regulation. It is important not only to detect H_2O_2 generation, but also to trace the dynamics of its intracellular content. Until recently, the study of the dynamics and localization of the H_2O_2 production in the course of diverse cellular processes was rather complicated. Usage of fluorescent probes, primarily DCFH-DA, has been the most common approach to detecting H_2O_2 in photodynamic reaction [4,5,14]. However, this approach is significantly limited due to low specificity of such probes toward hydrogen peroxide as well as inability to detect transient changes in the level of ROS both in the whole cell and in individual cell compartments.

The challenge can be addressed by using H_2O_2-sensitive fluorescent protein HyPer. This protein was created by cyclic permutation of the yellow fluorescent protein (cpYFP) and the fusion of its newly formed C- and N-termini with the H_2O_2-sensitive regulatory domain of the prokaryotic peroxide-specific transcription factor OxyR [15]. The photophysical properties of HyPer vary depending on the hydrogen peroxide concentration. H_2O_2-sensitivity is based on oxidation of the two thiol group in the OxyR domain with formation of disulfide bond. Oxidized HyPer is reduced in a glutaredoxin-mediated reaction. Conformational changes of HyPer due to its oxidation-reduction lead to changes in the shape of the fluorescence excitation spectrum. It allows ratiometric measurements of HyPer redox state and evaluation of H_2O_2 concentration in a complex intracellular medium in the absence of information on the sensor concentration. Due to the protein nature, HyPer can be directly expressed in target cells and addressed to various cellular compartments (cytoplasm, nucleus, mitochondria, etc.) [16].

HyPer is selectively oxidized by H_2O_2 and does not sensitive to such strong oxidizers as superoxide anion-radical, peroxynitrite and nitric oxide. As shown in experiments with the purified protein HyPer, the sensitivity of this sensor lies in the range of 25–250 nM H_2O_2 [15].

To date, HyPer has been successfully used to study H_2O_2 dynamics in cells and cell compartments under the action of a number of cytotoxic compounds [17,18], stimulation of growth factor receptors [19], skeletal muscle cells contraction [20]. In this work, the dynamics of the H_2O_2 content in cells after photodynamic treatment with photosensitizers of various structures was analyzed using HyPer.

2. Materials and Methods

2.1. Cell Line

The experiments were carried out on human cervical adenocarcinoma cell line HeLa-Kyoto HyPer-cyto expressing a genetically encoded sensor HyPer. The cell line was kindly provided by the Laboratory of Biophotonics of the Institute of Bioorganic Chemistry of the Russian Academy of Sciences. Cells were cultured in the DMEM medium (PanEco, Russia) with 10% (v/v) fetal calf serum (HyClone, USA) in 5% CO_2 at 37 °C.

2.2. Photosensitizers

The following photosensitizers were used: (i) chlorins Fotoditazin® (*N*-dimethylglucamine salt of chlorin *e6*; Veta-grand, Russia), and Radachlorin® (substance is Chlorin *e6* 80%, Purpurin 5 15% and Chlorin *p6* 5%; Rada-Pharma, Russia); (ii) phthalocyanines (NIOPIK, Russia) Photosens® (a mixture of di-, tri- and tetrasubstituted fractions of aluminum phthalocyanine, the number of sulfonic groups is 3.4); Holosens® (octachloride octakis[N(2-hydroxyethyl)-*N*,*N*,-dimethylammoniomethyl) zinc phthalocyanine) and Phthalosens® (a nonmetal sulphophthalocyanine with an average sulfonic group content of 2.5).

The photosensitizers are characterized by absorption and fluorescence in the red spectral region: the both chlorins absorb at 640–665 nm with fluorescence maxima at 650–675 nm [21,22], and the phthalocyanines has absorption maxima at 670–700 nm and emit at 685–710 nm [2,22].

2.3. Photoinduced Cytotoxicity Study

Photoinduced toxicity of the photosensitizers was stimulated using a LED light source providing a homogeneous light distribution in 96-well culture plates [23]. Cells were seeded in a plate at the density of 3×10^3 cells per well and allowed to attach overnight. The growth medium was then exchanged with 200 µl fresh serum-free medium containing 10 µg/ml photosensitizer and cells were incubated for 1 h. The medium was then exchanged with fresh growth medium and the plate was irradiated with a LED light source (615–635 nm). The dose of irradiation in individual wells was varied by duration of irradiation and ranged from 0.1 to 13.2 J/cm^2 at a power density of 20 mW/cm^2. Cell viability was assessed by MTT assay 24 h after irradiation. Cells were incubated with growth medium containing 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT reagent; Alfa Aesar, UK) for 4 h. The medium was then aspirated, and colored formazan crystals formed owing to the reduction of MTT reagent were dissolved in 200 µl dimethyl sulfoxide (PanEco, Russia). The absorbance in each well was measured at 570 nm with Synergy MX plate reader (BioTek, USA). Cell viability was calculated as a ratio of the optical density of irradiated to control (unirradiated) wells. Data analysis and calculation of half-lethal light dose LD_{50} were performed using the GraphPad Prism 6 software.

2.4. Microscopy Study of H_2O_2 Production

Monitoring of H_2O_2 production under photodynamic treatment was carried out using the Axiovert 200 M LSM 510 META laser scanning microscopy system (Carl Zeiss, Germany) equipped with a Plan-Apochromat 63 ×/1.40 Oil objective and incubator maintaining the cell culture conditions (5% CO_2 and 37 °C).

The day before the experiment, cells were seeded in 35 mm glass bottom Petri dishes at the density of 70×10^3 cells per dish. Before the photodynamic treatment, cells were incubated for 1 h in serum-free DMEM medium containing 10 µg/ml photosensitizer. The medium was then exchanged with a fresh growth medium.

The images of cells in transmitted light were obtained using halogen lamp and AxioCam MRc CCD-camera. For analysis of photosensitizers accumulation and intracellular localization, the fluorescence of dyes was excited by 633 nm HeNe laser and collected in the range of 650–710 nm.

Study of H_2O_2 production under photodynamic treatment was carried out on cells irradiated with neither halogen lamp nor HeNe laser. Cells were first scanned with the argon laser for 5 min in order to determine initial parameters of HyPer fluorescence and initial level of H_2O_2 in cells. Cells were then irradiated with 633 nm HeNe laser to induce the photodynamic response (or paused, in the case of control), and HyPer fluorescence parameters were further monitored.

The fluorescence of HyPer was excited sequentially at two wavelengths of the argon laser (458 nm and 488 nm), and collected in the range of 500–530 nm. The change in the H_2O_2 level was determined by the change in the I_{488}/I_{458} ratio, where I_{488} and I_{458} are the signals of HyPer fluorescence at the appropriate excitation wavelengths. Fluorescence images were recorded every 30 s for 5 min before irradiation and for 30–60 min after irradiation.

The photodynamic reaction was induced by 633 nm HeNe laser with laser output power at objective of 86 µW. The dose of irradiation equal to LD_{50} was used for each dye. Relevant doses were achieved by varying the exposure time.

Control cells ("dye control") were incubated in a growth medium with dyes, but were not irradiated with 633 nm HeNe laser. Additional experiments were conducted to identify the possible effect of irradiation of cells with the HeNe laser on the H_2O_2 production in the absence