Production of reactive oxygen species after photodynamic therapy by porphyrin sensitizers

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Abstract. The objectives of this study was to investigate the production of reactive oxygen species (ROS) after photodynamic therapy (PDT) *in vitro*. We examined second generation sensitizers, porphyrines ($TPPS_4$, $ZnTPPS_4$ and $PdTPPS_4$) and compared their effectivity on ROS generation in G361 cell line. Used porphyrines are very efficient water-soluble aromatic dyes with potential to use in photomedicine and have a high propensity to accumulate in the membranes of intracellular organelles like lysosomes and mitochondria. Interaction between the triplet excited state of the sensitizer and molecular oxygen leads to produce singlet oxygen and other ROS to induce cell death. Production of ROS was verificated by molecular probe CM-H_2DCFDA and viability of cells was determined by MTT assay. Our results demonstrated that $ZnTPPS_4$ induces the highest ROS production in cell line compared to $TPPS_4$ and $PdTPPS_4$ at each used concentration and light dose. These results consist with a fact that photodynamic effect depends on sensitizer type, its concentration and light dose.

Key words: Photodynamic therapy — Porphyrin sensitizer — Reactive oxygen species — Melanoma cells

Abbreviations: CM-H_2DCFDA, 5-(and 6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco′s modified Eagle′s medium; G361, human melanoma cell line; hp-β-CD, 2-hydroxypropyl-β-cyclodextrin; PBS, phosphate-buffered saline; PDT, photodynamic therapy; $PdTPPS_4$, palladium(II) *meso*-tetrakis(4-sulfonatophenyl)porphyrin; ROS, reactive oxygen species; $TPPS_4$, *meso*-tetrakis(4-sulfonatophenyl)porphyrin; $ZnTPPS_4$, zinc(II) *meso*-tetrakis(4-sulfonatophenyl)porphyrin.

Introduction

Photodynamic therapy (PDT) is a promising new modality for treatment of several types of cancer as well as of some chronic diseases. It utilizes a combination of sensitizer, visible light and molecular oxygen. Sensitizer in an excited triplet state initiates photochemical reactions *via* type I or II mechanism resulting in cell death and tumor necrosis. The type I mechanism involves electron transfer reactions to form free radicals and radical ions. These radical species are highly reactive and interact with molecular oxygen to form reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals. The type II mechanism results from an energy transfer from the triplet state of sensitizer to ground state molecular oxygen, leading to the generation of a very toxic singlet oxygen (Kochevar and Redmond 2000). The sensitizers used for PDT localize in various cytoplasmic membrane structures (mitochondria, plasma membrane, and lysosomes), but are not found in the nucleus. On the other hand, the cell nucleus is known to be a very sensitive target for ROS (Rosenkranz et al. 2000). The photodynamic dose depends on sensitizer concentration, oxygen concentration and fluence (effective light dose) applied and of course also on the intracellular location of the sensitizer (Kessel et al. 1997; Dougherty et al. 1998).

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The aim of this study was ROS and viability detection after PDT *in vitro* and comparison of their production in the presence of various porphyrin sensitizers for human melanoma cell line. Particularly, as sensitizers we used porphyrin derivative $TPPS_4$ (*meso*-tetrakis(4-sulfonatophenyl)porphyrin) and its zinc ($ZnTPPS_4$) and palladium ($PdTPPS_4$) complexes. These porphyrines are very efficient water-soluble aromatic dyes with high quantum yield of singlet oxygen and low aggregation tendency in neutral solutions (Mosinger et al. 2000). It is evident that the determination of the photochemical activity of the porphyrin sensitizers can play great importance for their application in cancer therapy.

Materials and Methods

Cell culture, sensitizers and irradiation conditions

The G361 human melanoma cells (ATTC, USA) were grown in 96-well microplates (10^4 cells/well), using cultivation Dulbecco´s modified Eagle´s medium (DMEM). Cell culture was stored in a CO_2 incubator (37°C, 5% CO_2) and incubated for 24 h. The cells in DMEM were then loaded with 1, 10 and 100 μmol/l sensitizer ($TPPS_4$, $ZnTPPS_4$, $PdTPPS_4$) and incubated for next 24 h. Excitation and emission wavelength of $TPPS_4$, $ZnTPPS_4$ and $PdTPPS_4$ are 413, 422, 410 nm and 645, 607, 606 nm, respectively. The cells were subsequently irradiated by a halogen lamp (24 V/250 W) at light doses of 25 and 50 $J \cdot cm^{-2}$. Irradiance was measured by Radiometer RK 2500 (Meopta Přerov, Czech Republic).

Microscopy

The intracellular ROS production was detected using nonfluorescent compound 5-(and 6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). Upon crossing the membrane, the compound undergoes deacetylation by intracellular esterases producing the non-fluorescent CM-H_2DCF, which quantitatively reacts with oxygen species inside the cell to produce the highly fluorescent dye CM-DCF. This compound remains trapped within the cell. Cells loaded with 10 μmol/l $ZnTPPS_4$ were treated with 5 μmol/l CM-H_2DCFDA for 30 min in darkness and then irradiated by light emitting diodes (LEDs; 418 nm, FWHM 15 nm, 3.3 $mJ \cdot cm^{-2} \cdot s^{-1}$) for 10 and 20 min at total light doses of 2 and 4 $J \cdot cm^{-2}$, respectively. Production of ROS was visualized by inverted fluorescent microscope Olympus IX 70, DP70 digital camera Olympus and Olympus MicroImage software.

ROS measurement

After incubation of cells with sensitizers DMEM was replaced by PBS with glucose (5.5 mmol/l) and cells were treated with 10 μmol/l CM-H_2DCFDA for 30 min at 37°C in darkness. The excess probe was washed out. Fluorescence of CM-DCF (excitation/emission: 495/530 nm) was recorded by Perkin–Elmer LS 50B luminescence spectrometer equipped with well plate reader accessory (Perkin–Elmer Corp., Norwalk, CT, U.S.A.).

Viability study

The phototoxicity of the sensitizers ($TPPS_4$, $ZnTPPS_4$, $PdTPPS_4$) in combination with irradiation against G361 cells was assayed by the MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma Aldrich). After photodynamic treatment, the cells were incubated at 37°C and 5% CO_2 for 24 h in fresh DMEM. Before start of the viability measurement, DMEM was replaced by PBS containing 5 mmol/l glucose and 2 mmol/l MTT (dissolved in PBS) and then the mixture was incubated for 3 h at 37°C and 5% CO_2. The MTT solution was carefully removed and 1 ml DMSO was added to liquidate a violet film. The absorbance of the prepared solution was measured in 96-well microplate reader Synergy HT at 570 nm and 690 nm. All MTT assays were repeated four times. Percent of cell viability of the test samples was related to contol samples (100 times average of test samples per average of control samples).

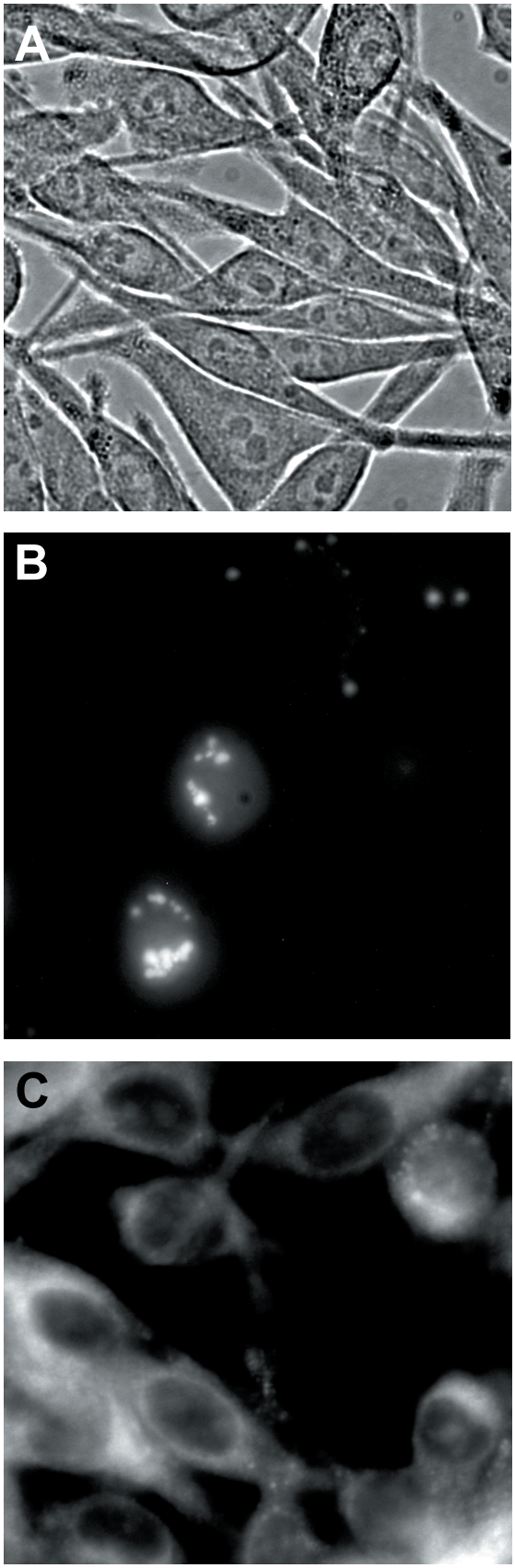
Results

Microscopy

Using molecular probe CM-H_2DCFDA in photosensitized cells we visualized production of ROS after 20 min of irradiation (Fig. 1). After 10 min of irradiation by LEDs we can observe CM-DCF fluorescence localized mainly in mitochondria and/or in lysosomes of G361 cells (Fig. 1B). After 20 min of irradiation the fluorescence of ROS is diffusely formed in whole cells (Fig. 1C). Microscopic image of live cells acummulated with 10 μmol/l $ZnTPPS_4$ was visualized in transmitted light (Fig. 1A).

ROS measurement

As demonstrated by the results reported in Fig. 2, our microplate assay shows the highest ROS generation in G361 cells by using 10 μmol/l $ZnTPPS_4$ at light doses of 25 and 50 $J \cdot cm^{-2}$ (Fig. 2A and B). Dose-dependent CM-DCF fluorescence demonstrated that ROS production in G361 cells acummulated with 10 μmol/l sensitizer rises only slightly with the increase in light dose above 25 $J \cdot cm^{-2}$ (Fig. 2C). This dependance can be observed for each used sensitizer and its concentration. So, in G361 cells it is possible to induce a demanded photodynamic effect already at light dose of 25 $J \cdot cm^{-2}$.



◄ **Figure 1.** Microscopic image of live G361 (A) cells loaded with 10 μmol/l $ZnTPPS_4$ (transmitted light). Cells were than treated with 5 μmol/l CM-H_2DCFDA and irradiated by LEDs for 10 (B) and 20 min (C, fluorescence images at excitation of CM-DCF).

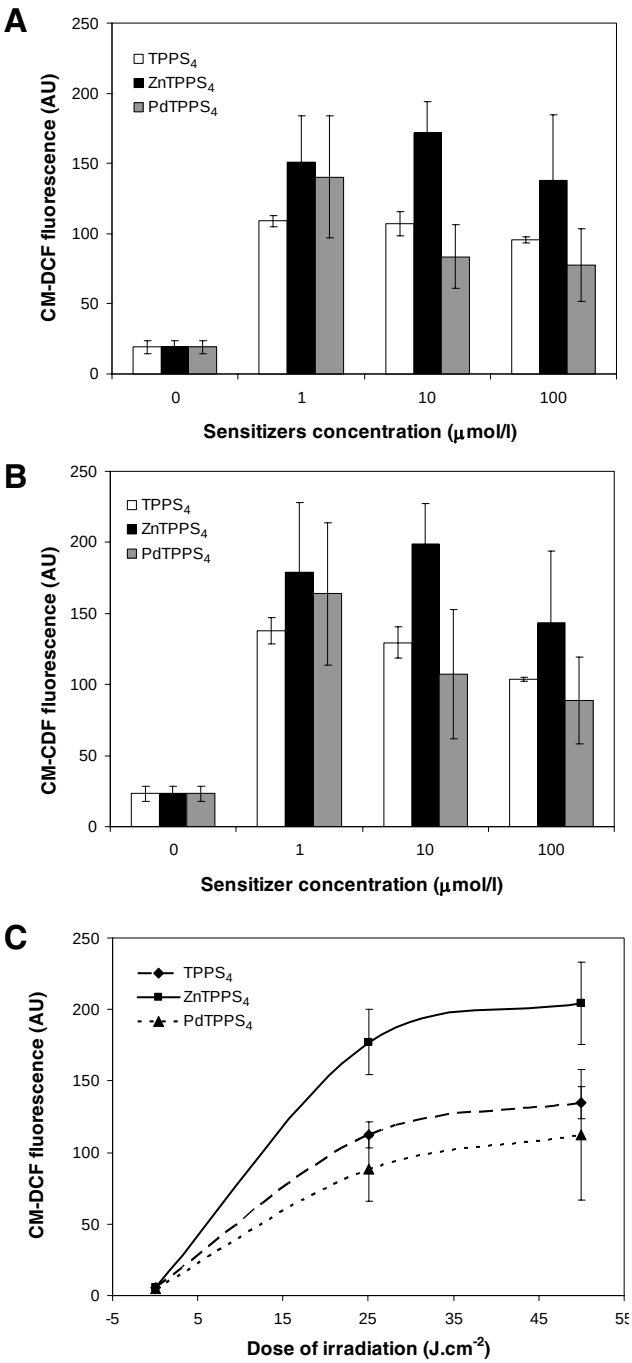


Figure 2. Dependence of ROS production in G361 cells on porphyrin sensitizer concentration after irradiation with light dose 25 J·cm^{-2} (A) and 50 J·cm^{-2} (B). Dose-dependent CM-DCF fluorescence (C) of G361 cells pretreated with 10 μmol/l sensitizers ($TPPS_4$, $ZnTPPS_4$, $PdTPPS_4$). Values represent mean ± S.E. from six independent experiments.

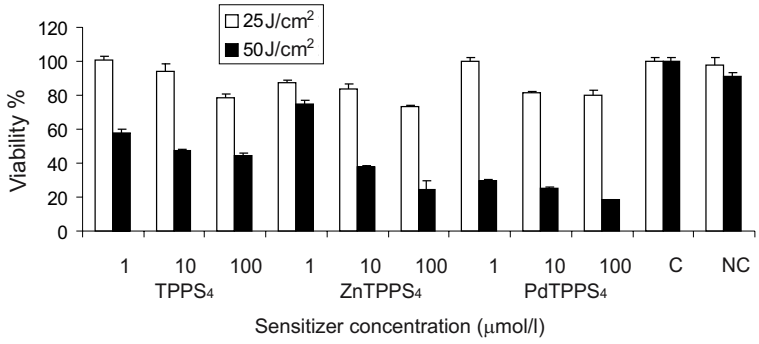


Figure 3. Dependence of G361 cell viability on the sensitizer (TPPS4, ZnTPPS4, and PdTPPS4) concentration and irradiation doses after photodynamic therapy. Bars C – control cells without irradiation and without sensitizers. Bars NC (negative controls) – cells without sensitizer with irradiation. Values represent mean ± S.E. from four independent experiments.

Viability study

Viability studies (Fig. 3.) show that the better phototoxic effect observed in G361 cells was obtained in the presence of the light dose of 50 J·cm^{-2} and at the sensitizer concentrations of 100 μmol/l.

Discussion

The photosensiting effects of porphyrins have been the subjects of extensive studies because of their potential use in photomedicine, e.g. in PDT (Kubat and Mosinger 1996; Kessel 2004). Porphyrins and porphyrin-related macrocycles are lipophilic and have a high propensity to accumulate in the membranes of intracellular organelles like lysosomes and mitochondria (Lam et al. 2001). In our previous study we demonstrated *in vitro* phototoxicity of two model sensitizers ($TPPS_4$ and $ZnTPPS_4$) in the presence or absence of cyclodextrin (HP-β-CD) on G361 cells. We proved their low cytotoxic and high phototoxic effect in visible region of the electromagnetic spectrum. The photodynamic effect was induced at light dose of 10 J·cm^{-2}, and $TPPS_4$ and $ZnTPPS_4$ concentration of 20 and 11.5 μmol/l, respectively. Moreover, we found that the most effective sensitizer is $ZnTPPS_4$ (concentration of 9.6 μmol/l) bound to HP-β-CD (Kolarova et al. 2003). In present study we monitored intracellular ROS formation as a measurement of the conversion of nonfluorescent CM-H_2DCFDA to fluorescent dye CM-DCF. We observed ROS formation in photosensitized G361 cells mainly in mitochondria and/or in lysosomes in the 10th minute from irradiation, followed whole cell distribution after next 10 min. The microplate assay demonstrated that $ZnTPPS_4$ induces the highest ROS production in cell line compared to $TPPS_4$ and $PdTPPS_4$. The highest ROS generation in G361 is shown by using 10 μmol/l porphyrine $ZnTPPS_4$ at each used concentration and light dose. In our previous paper we supposed (Kolarova et al. 2005) that usage of higher irradiation dose than 12.5 J·cm^{-2} and $ZnTPPS_4$ concentration higher than 10 μmol/l may improve the phototoxic effect in G361 cell lines. Now, we bring an evidence that higher dose of irradiation than 25 J·cm^{-2} does not have significant increase in ROS production in the tumorus cells. The $ZnTPPS_4$ concentration of 10 μmol/l seems to be the optimal to induce photodynamic effect in G361 cells and even furthermore higher dose can decrease the ROS production. Production of ROS generally corresponds to phototoxic effect. Higher ROS generation causes larger cell photodamage and consequently better phototoxic effect which is manifested later. ROS are formed in live cells promptly after irradiation, thus it is necessary to detect their production immediately after irradiation when cell viability is not yet affected as does our used sensitive method. The sensitizer loaded into the tumor cells generated singlet oxygen after irradiation resulting in cell death. Uptake of the sensitizer into tumor cells may also highly depend on the metabolic state of individual cells and time of incubation. We report the influence of sensitizers concentrations of 1, 10 and 100 μmol/l in combination with light irradiation dose at 25 and 50 J·cm^{-2} on the photodamage of G361 cells. Viability studies have shown, that photoxicity of $TPPS_4$ is less than phototoxicity of its metallocomplexes $PdTPPS_4$ and $ZnTPPS_4$. The best phototoxic effect observed in G361 cells was obtained in the presence of light dose of 50 J·cm^{-2} and at the $PdTPPS_4$ concentration of 100 μmol/l. Efficiency of PDT is affected by a number of factors including absorption spectrum of the photosensitizer, wavelength of the activation light, depth of the light penetration in the biological tissue, tissue answer on singlet oxygen. Cell death is thus confined to those illuminated areas in which there is an adequate presence and response of the sensitizing drug in the induction of nonreparable cell processes. High production

of ROS can induce significant oxidative damage of cellular biomolecules, including lipids, proteins and nucleic acids.

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