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In Vitro Study of Reactive Oxygen Species Production during Photodynamic Therapy in Ultrasound-Pretreated Cancer Cells

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Running title

ROS Production in Ultrasound-Pretreated Cancer Cells

Summary

Several recent studies bring evidence of cell death enhancement in photodynamic compound loaded cells by ultrasonic treatment. There are a number of hypotheses suggesting the mechanism of the harmful ultrasonic effect. One of them considers a process in the activation of photosensitizers by ultrasonic energy. Because the basis of the photodynamic damaging effect on cells consists in the production of reactive oxygen species (ROS), we focused our study on whether the ultrasound can increase ROS production within cancer cells. Particularly, we studied ROS formation in ultrasound pretreated breast adenocarcinoma cells during photodynamic therapy in the presence of chloroaluminum phthalocyanine disulfonate (ClAIPcS₂). Production of ROS was investigated by the molecular probe CM-H₂DCFDA. Our results show that ClAIPcS₂ induces higher ROS production in the ultrasound pretreated cell lines at a concentration of 100 μ M and light intensity of 2 mW/cm². We also observed a dependence of ROS production on photosensitizer concentration and light dose. These results demonstrate that the photodynamic effect on breast cancer cells can be enhanced by ultrasound pretreatment.

Keywords

Photodynamic therapy • Sonodynamic therapy • Phthalocyanine sensitizer • Reactive oxygen species

Abbreviations

ClAlPcS₂, chloroaluminum phthalocyanine disulfonate; CM-H₂DCFDA, 5-(and-6)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; LED, light- emitting diode; MCF7, human breast adenocarcinoma cell line; PBS, phosphate-buffered saline; PDT, photodynamic therapy; ROS, reactive oxygen species; SDT, sonodynamic therapy

Introduction

Sonodynamic therapy (SDT) is based on selective accumulation of sonosensitizing drugs in pathological tissues and subsequent activation of the sonosensitizers by ultrasound. SDT is used to induce cavitation, redistribution and disaggregation of the drugs, which in monomer forms produces a higher cytotoxic effect. In addition, the method has an advantage when compared to other physical treatment modalities in selective action because of the ability to focus ultrasonic energy into a small volume. Up to this day, several different chemical substances have been reported as potent sonosensitizers including porphyrins (Yumita et al. 1989, Umemura et al. 1990,), pheophorbide a (Umemura et al. 1990), dimethylformamide (Jeffers et al. 1995), merocyanine (Tachibana et al. 1999), piroxicam (Sakusabe et al. 1999), tenoxicam (Sakusabe et al. 1999), erythrosine B (Umemura et al. 1997, Hiraoka et al. 2006), rhodamine derivatives (Hiraoka et al. 2006), and phthalocyanines (Milowska and Gabryelak 2005). Considering that there are also a number of hypotheses trying to explain the principle of ultrasound action, it seems that the process includes several different physicochemical mechanisms. Umemura et al. (1996) suggest that a synergistic effect of ultrasound and sonosensitizers is due to photoexcitation of the drug by the sonoluminescence produced in collapsing cavitation. The mechanism of sonosensitization can also involve facilitated accumulation, redistribution and monomerization of the sonosensitizers (Misik and Riesz 1996, Miyoshi et al. 2001, Larina et al. 2005). Kessel et al. (1994) suppose that cytotoxicity is mediated largely by inertial cavitation. Inertial cavitation is a process where a gas bubble created by ultrasound in a liquid rapidly collapses, producing a shock wave with intense heat release (several thousand degrees Kelvin) (Worthington et al. 1997). The water molecules surrounding the cavitation decompose into their 'H and 'OH constituents (water pyrolysis), which either recombine, form H₂O, H₂O₂ and H₂, directly oxidize or reduce solute molecules, sonosensitizers or the biomolecules (Suslick 1990). Free

radical formation due to ultrasound action is strongly dependent on its threshold acoustic pressure at specific frequencies (Riesz and Kondo 1992).

Phthalocyanines belong to a second generation photosensitizers and are reported as being among the most effective drugs for photodynamic therapy (PDT) (Zavodnik *et al.* 2002). PDT is a promising therapy of malignant and nonmalignant diseases where the combined effect of photosensitizer, visible light and oxygen induces cell death. Upon absorption of appropriate light wavelengths, the photosensitizer is excited into a high-energy state, from which it is returned, accompanied by the transfer of an electron to adjacent molecules, referred to as a type I photochemical reaction, or energy to ground state of molecular oxygen, type II photochemical reaction (Henderson and Dougherty 1992, Nyman and Hynninen 2004). These processes produce reactive oxygen species (ROS) that are harmful to cells, such as singlet oxygen ¹O₂, superoxide radical anion O₂⁻⁻, hydroxyl radical ⁻OH, and hydrogen peroxide H₂O₂.

The aim of this study was to examine the effect of ultrasound exposure on ROS formation during subsequent PDT of breast adenocarcinoma cell line MCF7 in the presence of chloroaluminum phthalocyanine disulfonate ClAlPcS₂.

Methods

Cell culture and sensitizers

The MCF7 (human breast adenocarcinoma cell line) (ATTC, USA) was grown in 35 mm cell culture dishes $(3.3 \times 10^5 \text{ cells})$ in the presence of cultivation medium DMEM. Cell culture was stored in a humidified CO₂ incubator (37 °C, 5 % CO₂) for 24 h. The cells in DMEM were then loaded with 0, 1, 10 and 100 μ M phthalocyanine sensitizer ClAlPcS₂ prepared by Jan Rakusan at the Research Institute for Organic Syntheses in Rybitvi (Czech Republic) and incubated for subsequent 24 h.

Microscopy

Intracellular ROS production was detected using the nonfluorescent compound CM- H_2DCFDA (Invitrogen Corporation, USA). Upon crossing the membrane, the compound undergoes deacetylation by intracellular esterases producing the nonfluorescent 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 100 μ M ClAlPcS₂ were treated with 5 μ M CM- H_2DCFDA for 30 min in darkness and then irradiated by light emitting diodes (LEDs; 635 nm, FWHM 20 nm, 1 mW/cm²) for 10 min. Production of ROS was visualized by inverted fluorescence microscope Olympus IX 70 equipped with Olympus DP70 digital camera.

Ultrasound treatment

Ultrasound generator BTL–4000 (BTL, USA) with a transducer area of 4 cm², frequency 1 MHz and intensity 2 W/cm² was used for induction of the sonodynamic effect. The ultrasonic intensity output from the transducer was calibrated by radiation force balance against a primary standard and high performance hydrophone measurement system. After 24-h cell incubation with sensitizer, DMEM was replaced with PBS containing 5 mM glucose and 10 μ M CM-H₂DCFDA, and stored in a thermobox for 20 min at 37 °C. Then the extracellular probe was washed out by fresh glucose-enriched PBS and cells on 35 mm culture dishes were sonicated for 10 min at continuous rotation of 15 rpm and temperature of 37 °C.

ROS measurement

The assay using CM-H₂DCFDA is especially sensitive to the increased production of hydrogen peroxide or some of its downstream products (LeBel *et al.* 1992). Fluorescence of CM-DCF within cells adhered on a 35-mm culture cell dish (excitation and emission filter were 485/20 nm and 540/25 nm, respectively) was recorded as a kinetic measurement by Synergy HT reader equipped with a 5-mm reading probe from 4 places (BioTek, USA). The cells were continuously and homogeneously irradiated by 12 LEDs at a light intensity of 5×10^{-4} W/cm², 1×10^{-3} W/cm² and 2×10^{-3} W/cm². Other cells were exposed to a light irradiator consisting of 85 LEDs at a spatial homogeneous intensity of 20×10^{-3} W/cm² for 8 min and 20 s. Irradiance was measured by the radiometer system IL 1705 (International Light Technologies, USA). Fluorescence of CM-DCF was calibrated according to the corresponding fluorescence response of the probe to the additions of external H₂O₂. Briefly, cultured control cells in the absence of sensitizer were incubated with CM-H₂DCFDA. After removal of the extracellular probe by 2 mL of fresh PBS media, we recorded the increases of fluorescence signal in dependence on additions of 10, 25, and 50 µl of 20 mM H₂O₂, followed with 50 µL of 200 mM H₂O₂.

Data analysis

The data illustrate either representative traces or means \pm standard errors for 3 independent experiments. One-way analysis and Student's *t*-test were used for comparisons between experimental groups. Significance was set at *p* < 0.05.

Results

Microscopy

The morphology of the adherent human breast carcinoma cells MCF7 is shown in transmitted light in Fig. 1.A. Molecular probe CM-H₂DCFDA in photosensitized cells

visualized the ROS production sites after 10 min of irradiation. After this period we could observe CM-DCF fluorescence diffusely localized within the whole cell (Fig. 1B). The microscopic technique used is not able to recognize whether there are any subcellular structures that are excluded from the ROS production because of the high depth of focus.

ROS measurement

The effect of sensitizer ClAIPcS₂ concentration on ROS formation in ultrasound pretreated MCF7 cells was continuously monitored during application of PDT (Fig. 2). For the individual time course curve we calculated a rate of ROS production for the first four min using a linear regression analysis. The summary of the rate values for various sensitizer concentrations, light intensities and the effect of ultrasound pretreatment is presented in Fig. 3. Another experiment reported in Fig. 4 studied the total ROS production after the application of a 10 J/cm² light dose. The data showed that there is a significant difference of ROS production when we compared all the used sensitizer concentrations while a significant effect of sonication was expressed only for the application of a light intensity of 2×10^{-3} W/cm² or a total dose of 10 J/cm² in incubated cells with 100 μ M ClAIPcS₂.

Discussion

In the present study we examined whether the application of an ultrasound treatment can induce an increase of ROS within breast adenocarcinoma cell line MCF7 during PDT using ClAlPcS₂ as a sensitizer. We did not investigate the direct effect of the ultrasound energy on the ROS production accompanying collapse of cavitation microbubbles, such as pyrolysis of water vapor. One reason is the higher intracellular viscosity, which creates worse conditions for the creation of acoustic cavitations (Honda *et al.* 2004). On the other hand, when the microbubbles collapse, a part of the energy is transformed into light that can excite the photosensitizer. The phenomenon known as sonoluminescence has been investigated extensively. Under most conditions the intensity of sonoluminescence is very weak (Verall and Sehgal 1987). In addition, there is no evidence that the intensity is sufficient to excite phthalocyanine photosensitizers within cells. Our results showed a significant increase in ROS production, thus a significant effect of ultrasound pretreatment, for only MCF7 cells, which were incubated with the highest concentration of $ClAlPcS_2$ (100 μ M) and at a higher irradiation light intensity $(2 \times 10^{-3} \text{ W/cm}^2)$. Based on this fact, we suppose that there is a synergistic mechanism between SDT and PDT, which is involved in the ROS production within MCF7 cells and can be observed at the higher photodynamic conditions; sensitizer concentration and light intensity. It stands to reason that the total yield of ROS production affects the result of PDT. ROS overproduction causes severe cell damage and leads to cell death. In conclusion, we believe that the combination of SDT and PDT will bring medicine a new treatment modality for malignant and also nonmalignant diseases, although presently the mechanism of synergistic action is not fully explained. Miyoshi et al. 2001) showed that ultrasound induces monomerization of photosensitizers, which may increase the efficiency of PDT since only the monomers are photodynamically active. Moreover, when the photosensitizer accumulates in specific subcellular organelles, the ultrasound pretreatment can redistribute the photosensitizer to sites of higher vulnerability.

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Hana Kolářová, Department of Medical Biophysics, Faculty of Medicine, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic; e-mail: <u>kol@tunw.upol.cz</u>. **Fig. 1.** Microscopic images of live MCF7 cells loaded with 100 μ M ClAlPcS₂ in transmitted light (A, original image at 400x magnification). Fluorescence image of cells treated with 5 μ M CM-H₂DCFDA followed by 10 min irradiation shows the localization of its oxidation (B; fluorescence image at excitation of CM-DCF, original image at 400× magnification).

Fig. 2. ROS production traces reflect the dependence of CM-H₂DCF oxidation rates in MCF7 cells on phthalocyanine sensitizer concentration during continuous irradiation with light intensity of 2×10^{-3} W/cm². The representative traces were obtained from CM-H₂DCFDA pretreated cells incubated with 100 (trace a), 10 (trace b), 1 (trace c), and 0 (trace d) μ M of ClAlPcS₂ after ultrasound pretreatment. ^{*}The ROS production was expressed in concentration units of H₂O₂ according to the procedure described in detail in the Methods.

Fig. 3. Dependence of ROS production on concentration of ClAlPcS₂ during continuous irradiation with a light intensity of 5×10^{-4} W/cm² (white bars), 1×10^{-3} W/cm² (light gray bars), and 2×10^{-3} W/cm² (dark gray bars) in ultrasound- nonpretreated (bars without pattern) and pretreated MCF7 cells (bars with crosshatch pattern). *The rates of ROS production were calculated from the kinetic measurements for the first 4 min of irradiation. Their expression in concentration units of H₂O₂ is described in detail in the Methods section. Each value represents mean ± S.E. from 3 independent experiments. *Significant difference compared to lower light intensity (p < 0.05).

Fig. 4. Dependence of ROS production in MCF7 cells on concentration of ClAlPcS₂ and ultrasound pretreatment (bars with crosshatch pattern) after irradiation with a light intensity of 20×10^{-3} W/cm² for 8 min and 20 s resulting in a total dose of 10 J/cm². *The ROS production

was expressed in concentration units of H₂O₂ according to the procedure described in detail in the Methods. Each value represents mean \pm S.E. from 3 independent experiments. ⁺Significant difference of the ultrasound pretreatment in the presence of the same sensitizer concentration (p < 0.05). ^{#,‡}Significant differences compared to different sensitizer concentrations for ultrasound-nonpretreated and -pretreated cells, respectively (p < 0.05).



Fig. 1



Fig. 2



Fig. 3



Fig. 4