Physiol. Res. 56 (Suppl. 1): S27-S32, 2007

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

H. KOLÁŘOVÁ, R. BAJGAR, K. TOMÁNKOVÁ, E. KRESTÝN, L. DOLEŽAL, J. HÁLEK

Department of Medical Biophysics, Faculty of Medicine, Palacký University, Olomouc, Czech Republic

Received May 23, 2007
Accepted May 29, 2007
On-line available May 31, 2007

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 ($ClAlPcS_2$). Production of ROS was investigated by the molecular probe CM-H_2DCFDA. Our results show that $ClAlPcS_2$ induces higher ROS production in the ultrasound pretreated cell lines at a concentration of 100 μM and light intensity of 2 mW/cm^2. 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.

Key words

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

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 produce 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),

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

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 $^{\cdot}H$ and $^{\cdot}OH$ constituents (water pyrolysis), which either recombine, form H_2O, H_2O_2 and H_2, 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 $^1O_2$, superoxide radical anion $O_2^{\cdot-}$, hydroxyl radical $^{\cdot}OH$, and hydrogen peroxide H_2O_2.

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 $ClAlPcS_2$.

Methods

Cell culture and sensitizers

The MCF7 (human breast adenocarcinoma cell line) (ATTC, USA) was grown in 35 mm cell culture dishes (3.3×10^5 cells) in the presence of cultivation medium DMEM. Cell culture was stored in a humidified CO_2 incubator (37 °C, 5 % CO_2) for 24 h. The cells in DMEM were then loaded with 0, 1, 10 and 100 μM phthalocyanine sensitizer $ClAlPcS_2$ 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 μM $ClAlPcS_2$ were treated with 5 μM CM-H_2DCFDA for 30 min in darkness and then irradiated by light emitting diodes (LEDs; 635 nm, FWHM 20 nm, 1 mW/cm^2) 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 cm^2, frequency 1 MHz and intensity 2 W/cm^2 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 μM CM-H_2DCFDA, 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-H_2DCFDA is especially sensitive to the increased production of hydrogen

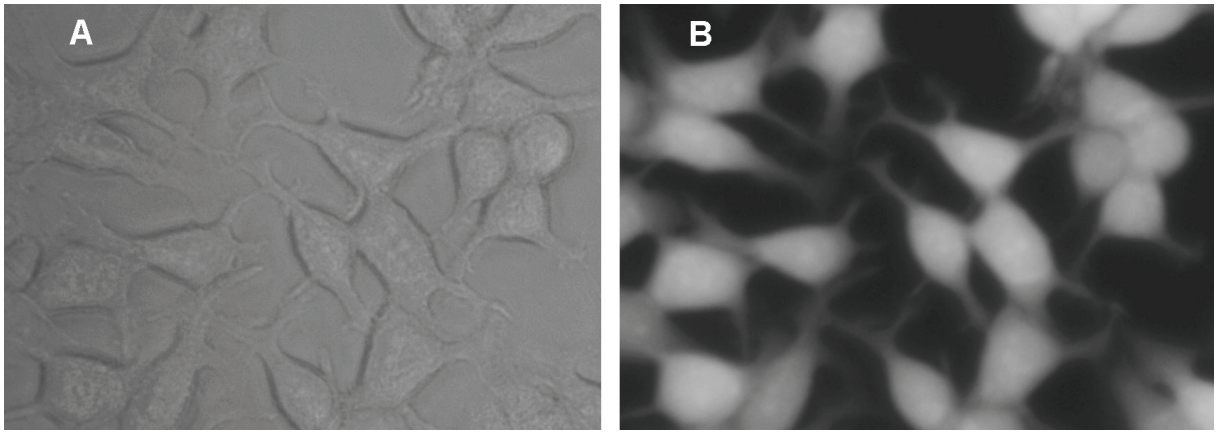

Fig. 1. Microscopic images of live MCF7 cells loaded with 100 μM $ClAlPcS_2$ in transmitted light (**A**, original image at 400x magnification). Fluorescence image of cells treated with 5 μM CM-H_2DCFDA followed by 10 min irradiation shows the localization of its oxidation (**B**; fluorescence image at excitation of CM-DCF, original image at 400× magnification).

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/cm^2, 1×10^{-3} W/cm^2 and 2×10^{-3} W/cm^2. Other cells were exposed to a light irradiator consisting of 85 LEDs at a spatial homogeneous intensity of 20×10^{-3} W/cm^2 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 H_2O_2. Briefly, cultured control cells in the absence of sensitizer were incubated with CM-H_2DCFDA. 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 H_2O_2, followed with 50 μL of 200 mM H_2O_2.

Data analysis

The data illustrate either representative traces or means ± 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-H_2DCFDA 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 $ClAlPcS_2$ 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/cm^2 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/cm^2 or a total dose of 10 J/cm^2 in incubated cells with 100 μM $ClAlPcS_2$.

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

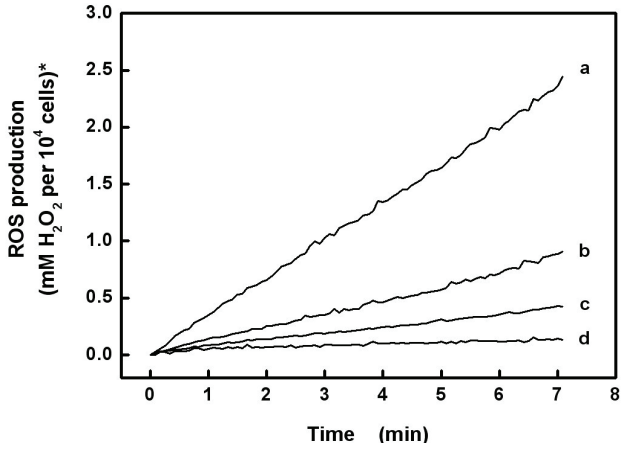

Fig. 2. ROS production traces reflect the dependence of CM-H_2DCF oxidation rates in MCF7 cells on phthalocyanine sensitizer concentration during continuous irradiation with light intensity of 2×10^{-3} W/cm^2. The representative traces were obtained from CM-H_2DCFDA pretreated cells incubated with 100 (trace a), 10 (trace b), 1 (trace c), and 0 (trace d) μM of $ClAlPcS_2$ after ultrasound pretreatment. The ROS production was expressed in concentration units of H_2O_2 according to the procedure described in detail in the Methods.

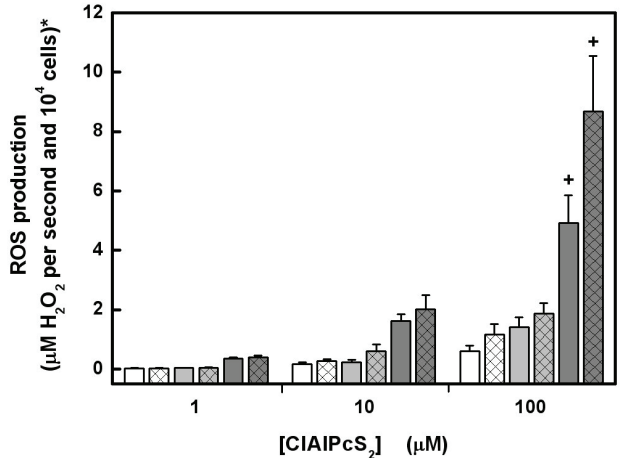

Fig. 3. Dependence of ROS production on concentration of $ClAlPcS_2$ during continuous irradiation with a light intensity of 5×10^{-4} W/cm^2 (white bars), 1×10^{-3} W/cm^2 (light gray bars), and 2×10^{-3} W/cm^2 (dark gray bars) in ultrasound- nonpretreated (bars without pattern) and pretreated MCF7 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 H_2O_2 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 ($p < 0.05$).

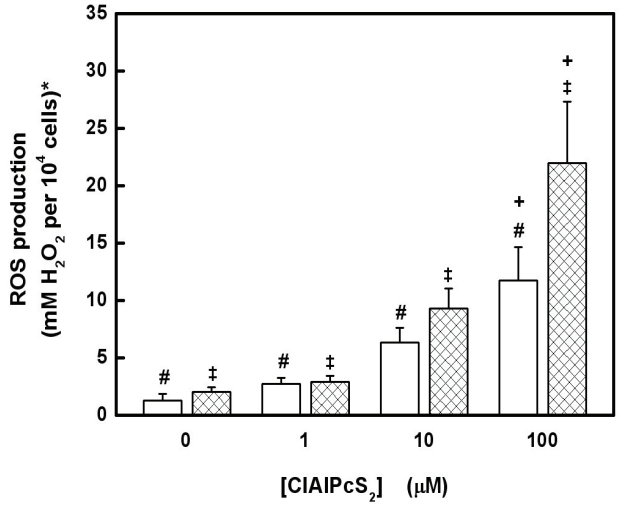

Fig. 4. Dependence of ROS production in MCF7 cells on concentration of $ClAlPcS_2$ and ultrasound pretreatment (bars with crosshatch pattern) after irradiation with a light intensity of 20×10^{-3} W/cm^2 for 8 min and 20 s resulting in a total dose of 10 J/cm^2. *The ROS production was expressed in concentration units of H_2O_2 according to the procedure described in detail in the Methods. Each value represents mean ± S.E. from 3 independent experiments. $^+$Significant difference of the ultrasound pretreatment in the presence of the same sensitizer concentration ($p < 0.05$). $^{\#,\ddagger}$Significant differences compared to different sensitizer concentrations for ultrasound-nonpretreated and -pretreated cells, respectively ($p < 0.05$).

MCF7 during PDT using $ClAlPcS_2$ 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 μM) and at a higher irradiation light intensity (2×10^{-3} 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.

Acknowledgements

This work was supported by the Ministry of Education of the Czech Republic MSM 6198959216.

Abbreviations

$ClAlPcS_2$, chloroaluminum phthalocyanine disulfonate; CM-H_2DCFDA, 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

References

HENDERSON BW, DOUGHERTY TJ: How does photodynamic therapy work? *Photochem Photobiol* **55**: 145–157, 1992.

HIRAOKA W, HONDA H, FERIL LBJR, KUDO N, KONDO T: Comparison between sonodynamic effect and photodynamic effect with photosensitizers on free radical formation and cell killing. *Ultrason Sonochem* **13**: 535–42, 2006.

HONDA H, KONDO T, ZHAO QL, FERIL LBJR, KITAGAWA H: Role of intracellular calcium ions and reactive oxygen species in apoptosis induced by ultrasound. *Ultrasound Med Biol* **30**: 683–692, 2004.

JEFFERS RJ, FENG RQ, FOWLKES JB, HUNT JW, KESSEL D, CAIN CA: Dimethylformamide as an enhancer of cavitation-induced cell lysis in vitro. *J Acoust Soc Am* **97**: 669–676, 1995.

KESSEL D, JEFFERS R, FOWLKES JB, CAIN C: Porphyrin-induced enhancement of ultrasound cytotoxicity. *Int J Radiat Biol* **66**: 221–228, 1994.

LARINA IV, EVERS BM, ESENALIEV RO: Optimal drug and gene delivery in cancer cells by ultrasound-induced cavitation. *Anticancer Res* **25**: 149–156, 2005.

LEBEL CP, ISCHIROPOULOS H, BONDY SC: Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem Res Toxicol* **5**: 227–231, 1992.

MILOWSKA K, GABRYELAK T: Synergistic effect of ultrasound and phthalocyanines on nucleated erythrocytes in vitro. *Ultrasound Med Biol* **31**: 1707–1712, 2005.

MISIK V, RIESZ P: Peroxyl radical formation in aqueous solutions of N,N-dimethylformamide, N-methylformamide, and dimethylsulfoxide by ultrasound: implications for sonosensitized cell killing. *Free Radic Biol Med* **20**: 129–138, 1996.

MIYOSHI N, TAKESHITA T, MISIK V, RIESZ P: Monomerization of photosensitizers by ultrasound irradiation in surfactant micellar solutions. *Ultrason Sonochem* **8**: 367–371, 2001.

NYMAN ES, HYNNINEN PH: Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy. *J Photochem Photobiol B* **73**: 1–28, 2004.

RIESZ P, KONDO T: Free radical formation induced by ultrasound and its biological implications. *Free Radic Biol Med* **13**: 247–270, 1992.

SAKUSABE N, OKADA K, SATO K, KAMADA S, YOSHIDA Y, SUZUKI T: Enhanced sonodynamic antitumor effect of ultrasound in the presence of nonsteroidal anti-inflammatory drugs. *Jpn J Cancer Res* **90**: 1146–1151, 1999.

SUSLICK KS: Sonochemistry. *Science* **247**: 1439–1445, 1990.

TACHIBANA K, UCHIDA T, OGAWA K, YAMASHITA N, TAMURA K: Induction of cell-membrane porosity by ultrasound. *Lancet* **353**: 1409, 1999.

UMEMURA K, YUMITA N, NISHIGAKI R, UMEMURA S: Sonodynamically induced antitumor effect of pheophorbide a. *Cancer Lett* **102**: 151–157, 1996.

UMEMURA S, KAWABATA K, SASAKI K: In vitro and in vivo enhancement of sonodynamically active cavitation by second-harmonic superimposition. *J Acoust Soc Am* **101**: 569–577, 1997.

UMEMURA S, YUMITA N, NISHIGAKI R, UMEMURA K: Mechanism of cell damage by ultrasound in combination with hematoporphyrin. *Jpn J Cancer Res* **81**: 962–966, 1990.

VERALL RE, SEHGAL CM: Sonoluminescence. *Ultrasonics* **25**: 29–30, 1987.

WORTHINGTON AE, THOMPSON J, RAUTH AM, HUNT JW: Mechanism of ultrasound enhanced porphyrin cytotoxicity. Part I: A search for free radical effects. *Ultrasound Med Biol* **23**: 1095–1105, 1997.

YUMITA N, NISHIGAKI R, UMEMURA K, UMEMURA S: Hematoporphyrin as a sensitizer of cell-damaging effect of ultrasound. *Jpn J Cancer Res* **80**: 219–222, 1989.

ZAVODNIK IB, ZAVODNIK LB, BRYSZEWSKA MJ: The mechanism of Zn-phthalocyanine photosensitized lysis of human erythrocytes. *J Photochem Photobiol B* **67**: 1–10, 2002.

Corresponding author

H. Kolářová, Department of Medical Biophysics, Faculty of Medicine, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic. E-mail: kol@tunw.upol.cz