ORIGINAL CONTRIBUTIONS

O Experimental ONCOLOGY

PHOTO-OXIDATIVE ACTION IN CERVIX CARCINOMA CELLS INDUCED BY HPD — MEDIATED PHOTODYNAMIC THERAPY

*J. Saczko[1, *], W. Skrzypek[2], A. Chwiłkowska[1], A. Choromańska[1], A. Poła[4], A. Gamian[1, 3], J. Kulbacka[1]*

[1]*Department of Medical Biochemistry, Silesian Piasts University of Medicine, 50-368 Wroclaw, Poland*
[2]*St. Hedwigs Hospital, 55-100 Trzebnica, Poland*
[3]*Department of Immunology of Infectious Diseases, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland*
[4]*Department of Biophysics, Silesian Piasts University of Medicine, 50-368 Wroclaw, Poland*

Photodynamic therapy leads to oxidative stress through the generation of free radicals. Oxidative stress causes damage to cellular macromolecules such as nucleic acids, proteins and lipids. *Aim:* To examine the hematoporphyrin derivative (HpD) — mediated photodynamic effect on cervical adenocarcinoma cell line HeLa. *Methods:* The HpD localization in HeLa cells was analyzed by confocal microscopy with epi-fluorescence system. Lipid peroxidation (LPO) was estimated by measurement of the concentration of malondialdehyde, protein degradation — by modified Ellman's method, superoxide dysmutase (SOD) — using Ransod Kit. The expression of inducible nitric oxide synthase (iNOS) was detected by immunocytochemical staining. *Results:* The HpD was distributed all over the cytoplasm with preferential localization in the inner side of the plasma membrane and around the nuclear envelope. The process of photosensitizer distribution was time dependent. PDT-HpD increased the level of malonodialdehyde (MDA), SOD activity and the expression of iNOS in HeLa cells. However, PDT induced the decrease in the level of protein-associated thiol groups. *Conclusions:* Our study showed the important role of PDT-mediated oxidative stress in HeLa cells. HpD-PDT might be alternative and less invasive approach for treatment of patients with cervical cancer resistant for standard chemotherapy and radiotherapy.
Key Words: **cervical adenocarcinoma, photodynamic treatment, HpD, lipid peroxidation, thiol groups, nitric oxide synthase, superoxide dismutase.**

Cervical carcinoma is the most frequent cause of death from cancer in women from developing countries and areas where social changes and urbanization may increase HPV infection among young generations [1]. Therefore, new diagnostic and therapeutic techniques with significantly improved sensitivity and specificity are required. Photodynamic therapy (PDT) is still a developing method in the arsenal of anticancer therapy, based on photosensitizer and the visible light. This treatment leads to the generation of reactive oxygen species (ROS), such as singlet molecular oxygen, hydroxyl radicals, and/or superoxide anions [2]. The use of PDT as cancer therapy is particularly advantageous because of its intrinsic dual selectivity. The photosensitizer localizes in the malignant tissue and the light is also spatially focused on the lesion [3].

In PDT, photosensitizer is accumulated in tumor cells, which is followed by irradiation with visible light. The oxidative stress and ROS are factors which initiate the cell death via apoptosis or necrosis in PDT [4, 5]. Although PDT is designed to cause a cytotoxic reaction in tumor tissue, a post-PDT response involving inflammatory, innate, and adaptive immune reactions is envisioned to assist in successful eradication of residual surviving tumor cells [2, 6]. The reactions of free radicals with unsaturated lipids and proteins in membranes may directly cause alterations of membrane function. Mitochondrial photosensitization is of particular interest because of the key role performed by mitochondria in cell metabolism as well as in the regulation of normal cell functions [7, 8].

Therefore, the intracellular accumulation of the photosensitizers is one of the most important factors to determine the efficacy of the PDT [9, 10]. Depending on their physicochemical properties and their uptake mechanism, sensitizers can achieve different intracellular concentrations and localize in different subcellular compartments. Cell death mechanism after PDT is determined by the preferential localization of a sensitizer in target organelles [11, 12].

The effective antioxidant defence system requires an increase in antioxidant enzyme activity, not only to eliminate the initial radicals, but also the more toxic products of spontaneous free-radical reactions [13]. One of the most important antioxidant enzymes is superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion O_2^- to O_2 and H_2O_2. SOD can partially prevent the photodestruction caused by PDT [14, 15].

Nitric oxide (NO) is generated by the oxidation of arginine, a reaction catalyzed by the NO synthase (NOS) enzyme. NO has become recognized as a major effector molecule in diverse array of physiologic and pathologic processes [16–19]. A major mechanism for cell injury produced by NO *in vivo* is due to its diffusion-limited reaction with superoxide to form peroxynitrite [20]. Recently, NO production was demonstrated in drug-treated photosensitized tumor cells [21]. NO plays an important dualistic role in malignant cells after PDT. In tumors producing high levels of NO, the PDT-induced reduction in tumor blood flow, vascular occlusion and consequent

Abbreviations used: HpD – hematoporphyrin derivative; iNOS – inducible nitric oxide synthase; LPO – lipid peroxidation; MDA – malonodialdehyde; NO – nitric oxide; PDT – photodynamic therapy; ROS – reactive oxygen species; SOD – superoxide dismutase.

ischemia may be diminished, while the inflammatory reaction triggered by PDT may be suppressed. On the other hand, elevated NO levels may maintain vessel dilation during PDT light treatment, which can diminish the decrease in tumor oxygenation and sustain in this way the oxygen-dependent generation of phototoxic damage. PDT induces inflammation, afterwards the tumor cells might activate macrophages to produce iNOS, which results in excessive NO release [17, 22, 23].

In the current study we applied PDT on *in vitro* model of human cervical adenocarcinoma HeLa cells. The aim of this study was to evaluate HpD-mediated photodynamic effect on HeLa cells with the particular focus on oxidative stress factors, such as LPO, the level of –SH groups, SOD activity and immunocytochemical determination of inducible NO synthase.

MATERIALS AND METHODS

Cell culture. HeLa cell line was purchased from Institute of Immunology and Experimental Therapy, Polish Academy of Sciences. The cell line was routinely propagated in monolayer cultures in Dulbecco's modified Eagles' medium (Sigma) supplemented with 10% fetal bovine serum and glutamine with penicillin/streptomycin (Sigma) in 25 cm^2 Falcon flasks. The cells were maintained in a humidified atmosphere at 37 °C and 5% CO$_2$.

Photodynamic treatment. The cells were treated with 30 µg/mL HpD (Hematoporphyrin «D», Porphyrin Products Inc., USA) in complete media, for 4 h in the dark. Then cells were irradiated with a light dose of 3 and 6 J/cm^2 using lamp (10 mW/cm^2) (OPTEL, Opole, Poland) with polarized light and red filter (632.8 nm). After irradiation the cells were incubated in a humidified atmosphere at 37 °C and 5% CO$_2$ for 0, 3 and 18 h. Control cells with HpD were incubated in the same conditions.

The localization of HpD. Cells were grown on cover glasses (24 x 24 mm, Thermo Scientific), and incubated for 4 h with HpD at the concentration of 30 µg/mL. After incubation, the cells were washed in PBS and then fixed in 4% formalin buffer, washed 2 times in PBS and water (Aqua Pro Injectione, Polpharma), then examined by confocal microscopy with epi-fluorescence system (Nikon Eclipse TE2000-E) using filters with an excitation wavelength of 528–553 nm and emission wavelength of 578–633 nm.

Lipid peroxidation. The LPO was determined by measurement of the final product of fatty acid peroxidation — MDA according to modified procedure described by Kulbacka *et al.* [24]. The cells after photodynamic treatment (as described above) *in vitro* were trypsinized (0.25% Trypsin-EDTA), washed twice in PBS, and re-suspended in 200 µl of PBS. The concentration of MDA was quantified spectrophotometrically (λ = 535 nm) using a set of MDA standards.

Proteins damage. The evaluation of protein degradation is based on modified Ellman's method. This method uses reaction of DTNB acid with thiol groups of proteins. The level of –SH groups was measured

spectrophotometrically on the basis of the absorbance at the wavelength of 412 nm [25].

Determination of SOD activity. After photodynamic treatment the cells were trypsinized and washed twice in PBS. Then the cells were suspended in 50 mM PBS, pH 7, with a mixture of protease inhibitors (Complete Mini EDTA-free, Roche). The total intracellular SOD activity was measured using a Ransod assay (Randox Laboratories Ltd., Antrim, United Kingdom) according to the manufacturer's protocol.

Expression of iNOS. The cells were plated into 8-dip glasses (Nunc). For immunocytochemical detection of iNOS rabbit polyclonal anti-iNOS antibodies were used (1:100, Santa Cruz, USA). Formalin-fixed (4%) cells were immunostained by DAKO LSAB 2 kit. The % of stained cells was determined by counting 100 cells in three randomly selected fields, performed by two independent investigators. Cells were considered as positive if staining was observed in > 5% of cells. The intensity of immunocytochemical staining was evaluated as follows: (–) negative, (+) weak, (++) moderate and (+++) strong. Positive and negative controls were included. Two independent experiments were performed for each PDT combination.

Statistical analysis. Normality of continuous variables was checked by the Shapiro-Wilk test. The significance of the difference between mean values of different groups of cells in comparison to control untreated cells was assessed by Student's *t*-test, values of $p \leqslant 0.05$ were considered significant.

RESULTS

Localization of HpD. The intracellular distribution of HpD monitored after 4 h of treatment is presented on Fig. 1A and B. We could observe the dye diffused throughout the cytoplasm. After 4 h the most intensive signal was detected next to the inner side of the plasma membrane (Fig. 1, *a, b*).

The evaluation of LPO. After PDT, the level of LPO increased in cells that received the highest dose of irradiation (6 J/cm^2) at all pre-incubation periods. Immediately post PDT and 3 h after it the concentration of MDA was on the same level (0.4–0.6 µM/L). 18 h after photodynamic treatment we have observed a significant decrease of MDA level, which was below the control (Fig. 2, *a*).

The evaluation of protein damage. The proteins degradation was observed for all incubation time points after PDT application, but mainly immediately after PDT and 3 h after it. After 18 h of irradiation, we observed double rise of the protein-associated –SH groups concentration in comparison to control untreated cells (Fig. 2, *b*) ($p < 0.05$).

SOD activity. We observed that SOD activity achieved the highest level (to about 20 U/mg of protein) directly after irradiation and after 18 h post PDT. However 3 h post irradiation the SOD activity decreased to 14 U/mg of protein (Fig. 2 C). The highest value of the enzyme activity was observed for the cells that received the highest dose of irradiation (6 J/cm^2). The values
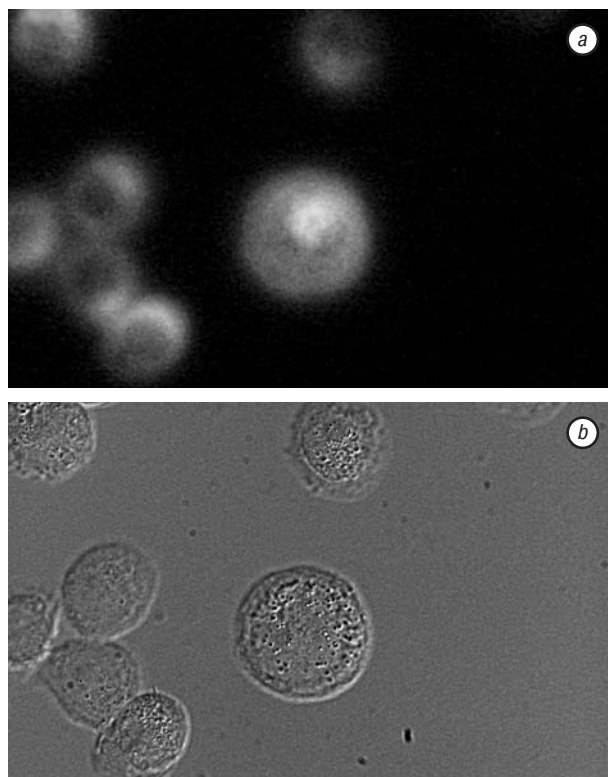
Fig. 1. Confocal microscopy of the distribution of HpD in HeLa cells incubated for 4 h; *a*, fluorescent microscopy, *b*, brightfield image (x1000)

of enzyme activity were statistically significant only for PDT dose of 6 J/cm^2 in comparison to control cells (p < 0.015).

Immunocytochemistry of iNOS. The results of iNOS reaction are presented in the Table 1 and on the Fig. 3. The most intensive immunocytochemical reaction was observed 18 h after HpD-PDT (Fig. 3, *c* and *d*). For both irradiation doses, directly after PDT only individual cells showed the reaction (Fig. 3, *b*), and in 3 h after PDT 50% of cells were stained (Table 1).

Table 1. The effect of PDT with HpD on iNOS expression in HeLa cells.

PDT dose		Incubation time after PDT, h	Staining intensity	Immunopositive cells,%
HpD, µg/ml	Irradiation, J/cm^2			
0	0	0	–	0
30	0	0	–	0
30	3	0	– / +	single cells
	6		– / +	single cells
30	3	3	+	50
	6		+	48
30	3	18	+ +	98
	6		+ +	80

DISCUSSION

The cellular response to PDT is monitored by numerous parameters such as the intracellular localization of the drug, the physical and chemical properties of the photosensitizer and the cell type. Depending on the photosensitizer used, photodynamic treatment has been shown to induce lesions in membranes, mitochondria, lysosomes or DNA [26]. Our *in vitro* studies showed that the intracellular accumulation of HpD was time dependent in HeLa cells. This is similar to our previous study where we showed a time dependent increase of accumulation of the fluorescent dye (Ph, Photofrin®) in other malignant cell lines (A549, MCF-7,
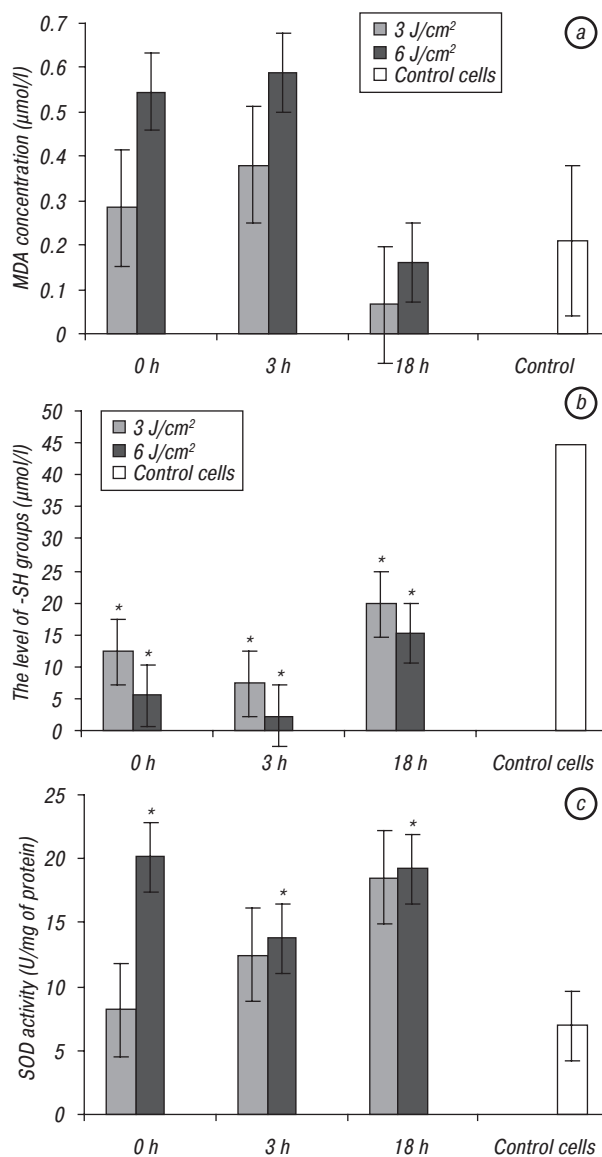

Fig. 2. The evaluation of oxidative stress markers in HeLa cell after photodynamic treatment: *a,* The production of malondialdehyde by HeLa cells after PDT with HpD. The level of MDA was quantified at once after photodynamic reaction and after 3 and 18 h; *b,* The level of protein-associated -SH groups in HeLa cells after photodynamic treatment with PDT. The protein degradation was quantified after photodynamic reaction after 0, 3 and 18 h; *c,* The changes of SOD activity in HeLa cells after photodynamic treatment with HpD. Control cells were not irradiated and treated with HpD. Error bars shown are means ± SD for $n = 3$ ($n = 4$ for C); *p < 0.05.

Me45). Our data showed that staining was mainly localized in intracellular compartments, but in several cases also in mitochondrial membranes [27]. Other study showed that about 15% of HpD was localized in the nucleus of two cell lines: human glioma (BMG-1) and sqamous carcinoma (4451) cell lines [28]. However, localization in the nucleus may not always lead to cell death and can induce mutations leading to carcinogenesis [29].

We observed that PDT- HpD induced the oxidative stress in HeLa cells. The light dose and the time of incubation after PDT affected significantly dynamic changes in the level of oxidative stress. The photodynamically-generated increase of MDA concentration with additional
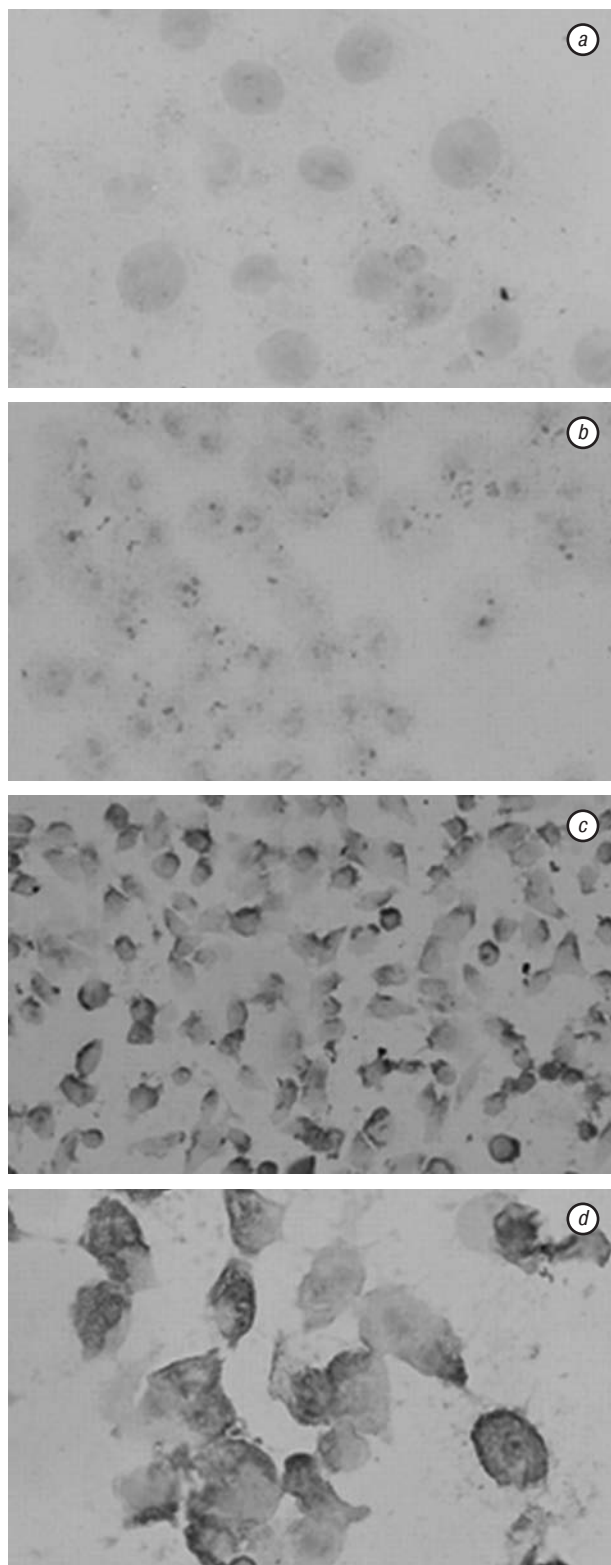
Fig. 3. The expression of iNOS in *(a)* control HeLa cells (x1000); *(b)* immediately after PDT (the radiation dose: 6 J/cm², the HpD concentration: 30 µg/mL) (x400); *(c)* 18 h post PDT (the light dose: 3 J/cm², the HpD concentration: 30 µg/mL) (x400); *(d)* 18 h post PDT (the light dose: 6 J/cm², the HpD concentration: 30 µg/mL) (x1000)

protein damage (-SH groups) were observed directly after PDT. Peroxidation of membrane lipids is known to disrupt membrane structure and induce loss of function which could lead to cell death. Our investigation confirms earlier PDT studies on different cell lines, showing

that after PDT the LPO level was increased [30, 31]. Our results showed that LPO decreased with the increase of incubation time after PDT and negatively correlated with the thiol groups' concentration.

The oxidative stress induced by PDT causes damage to cellular macromolecules such as proteins, lipids and nucleic acids. Among these targets, the peroxidation of lipids is particularly damaging because the formation of LPO products leads to a facile propagation of free radicals and being accompanied with the oxidation of thiol groups leads to the membrane disintegration [13].

Our results show that increasing incubation time after PDT induces the rise of iNOS expression. Inducible NOS is a target enzyme because it is not dependent on calcium concentration. Additionally, iNOS produces NO much more efficiently than eNOS or nNOS, and this efficiency allows effective treatment at low levels of transgenic expression [32]. NO may play a role in tumor development, tumor immune responses, metastasis and apoptosis [23]. It has been demonstrated that NO can modulate the effect of cancer therapies that have an oxidative mechanism [33, 34].

We showed that in photodynamically treated HeLa cells decrease of LPO induced rise of SH-groups level and slow increase in iNOS activity. The current results indicate the PDT-HpD influence on generation of ROS, which are a signal for development of apoptosis or necrosis in human cervical carcinoma cells [4, 9]. Bar *et al.* [35] investigated the response of OvBH-1 cells to Ph II-PDT. These cells demonstrated pronounced resistance to PDT-induced apoptosis, which was visualized by the lack of remarkable changes in their morphological features, DNA integrity and expression of apoptosis related proteins [35]. In the present study the total intracellular SOD activity in the cells after PDT was examined. Our results showed that HpD-PDT induced the increase of SOD activity. SOD is a protective enzyme and reduces oxidative stress. Inhibition of SOD causes accumulation of cellular O_2^- and leads to free-radical-mediated damage to mitochondrial membranes, the release of cytochrome c from mitochondria and apoptosis of the cancer cells [15, 36, 37]. Differences in cell susceptibility to PDT may depend upon a protective mechanism, such as MnSOD gene induction [38]. Other authors used ZnTM-2(3,4)-PyP⁴⁺, that can act as a photosensitizer with efficacy comparable to that of HpD in preventing cell proliferation and causing cell death *in vitro*. They applied this photosensitizer to colon adenocarcinoma cells which induced LPO, membrane permeability, oxidative DNA damage, and the activities of SOD, catalase, glutathione reductase, and glutathione peroxidase [39]. Kasugai *et al.* [37] checked the novel water-soluble Fe-porphyrins with SOD activity in Walker 256, H-4-II-E and FR cells. It was shown that cell death can be induced by Fe-porphyrins that affect SOD mimic. The authors' results suggest that for the SOD mimic, O_2^- may be applied as a target molecule to provoke selectively cancer cell death. They intend new metalloporphyrins possessing SOD activity as a new class of anticancer agents. Other investigations indicate that targeting SOD may be a promising approach for se-

lective killing of cancer cells, and that mechanism-based combinations of SOD inhibitors (2-Metoxyestradiol) with free-radical-producing agents may have clinical applications [15].

Our study showed that PDT induced the increase of SOD activity and LPO and decrease of the level of thiol groups.

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