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On the mechanism of reactive oxygen species generation in tumour cells subjected to the phototoxic action of haematoporphyrin derivative: effect of heating

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Abstract. The main aims of the study were: (1) to enrich the existing knowledge on the mechanism of H_2O_2, superoxide ($O_2^{-\bullet}$), and protein peroxides (PPO) formation in tumour cells subjected to photodynamic therapy (PDT) with haematoporphyrin derivative (HPD) and (2) to explain the stimulatory effect of heat stress on the generation of $O_2^{-\bullet}$ (a precursor of H_2O_2 and a very reactive hydroxyl radical) in HPD–PDT-treated cells. Experiments were performed on Ehrlich ascites carcinoma (EAC) cells, which were loaded with HPD in phosphate-buffered saline and then irradiated with red light at 630 nm in the same buffer. Studies showed that photoexcited HPD itself, i.e. in the absence of photooxidizable biomolecules, is a poor source of H_2O_2 and oxygen radicals, and that in tumour cells subjected to PDT with HPD the generation of H_2O_2 and $O_2^{-\bullet}$ could be largely explained by (i) photooxidation of certain cellular constituents (NAD(P)H), (ii) an increase in the activity of xanthine oxidase (XOD), and (iii) a photodamage to mitochondria. Besides, it was found that in cellular proteins the HPD-photosensitized oxidation of aromatic amino acids is responsible for the generation of H_2O_2 and PPO. Our data suggest that upon HPD–PDT the mild hyperthermia (ca 44 °C) produced by photoirradiation may enhance its tumouricidal effect via the stimulation of $O_2^{-\bullet}$ formation; it was found that a rise in the temperature from 30 to 44 °C strongly (by ca 2.5-fold) enhanced the generation of $O_2^{-\bullet}$ in EAC cells, which correlated well with an increase in the rate of their HPD-photosensitized killing. Studies showed that the intensification of $O_2^{-\bullet}$ formation is mediated by the stimulatory effects of heating on the activity of XOD as well as the production of this radical by the respiratory chain of mitochondria. Nevertheless, the obtained results indicate that severe hyperthermia (at temperatures >45 °C) could induce, contrary to mild hyperthermia, a reduction in the efficiency of HPD–PDT due to suppression of the activity of XOD in tumour cells.

Key words: photodynamic therapy, tumour, reactive oxygen species, xanthine oxidase, hyperthermia.

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Abbreviations: AAP = amino acid peroxides; ANM = antimycin A; AP = allopurinol; BSA = bovine serum albumin; CAT = catalase; DTNB = 5,5'-dithiobis-2-nitrobenzoic acid; EAC = Ehrlich ascites carcinoma; His = histidine; HPD = haematoporphyrin derivative; H_2O_2 = hydrogen peroxide; LD_{50} = the light dose at which 50% of the cells were stained by trypan blue; NADH = reduced nicotinamide adenine dinucleotide; NADPH = reduced nicotinamide adenine dinucleotide phosphate; NBT = nitro blue tetrazolium; $^1O_2$ = singlet oxygen; $O_2^{-\bullet}$ = superoxide radical; $OH^\bullet$ = hydroxyl radical; PBS = phosphate-buffered saline; PDT = photodynamic therapy; PPO = protein peroxides; PrSH = protein-bound sulphydryl groups; PS = photosensitizer; ROS = reactive oxygen species; SE = standard error; SOD = superoxide dismutase; TB = trypan blue; TCA = trichloroacetic acid; Trp = tryptophan; Tyr = tyrosine; XDH = xanthine dehydrogenase; XO = xylenol orange; XOD = xanthine oxidase; XOR = xanthine oxidoreductase.

INTRODUCTION

Photodynamic therapy (PDT) involves administration of a tumour-localizing agent (photosensitizer, PS) followed by activation of the agent by light (usually from a laser) at a specific wavelength. This results in a sequence of photochemical and photobiological processes, which cause irreversible photodamage to tumour tissues. Preclinical and clinical studies conducted worldwide over a 30-year period have established PDT as a useful approach for treatment of some tumours [1]. Haematoporphyrin derivative (HPD), commercially known as Photofrin, is still the most widely used PS in PDT of malignancies. Studies on the mechanism of PDT showed that HPD accumulates extensively in membrane structures of tumour cell and upon photoexcitation is able to generate singlet oxygen ($^1O_2$), a highly active oxidant. The rapid tumouricidal response observed following HPD–PDT results from oxidative injury to both the treated tumour cells and the exposed vasculature [2]. Subcellular targets of HPD–PDT include the plasma membrane, mitochondria, cytoskeletal proteins, DNA, cytosol, and mitochondrial enzymes [2–4]. Photosensitization by HPD can also induce programmed cell death or apoptosis [5]. It was discovered that local immune reactions, e.g. macrophages, could be also responsible for the tumour necrotizing effect induced by HPD–PDT [6]. Although to date PDT with HPD has already proved its effectiveness in the treatment of cancer, the molecular mechanisms by which this therapy destroys tumour cells as well as its optimal physical parameters are still incompletely understood. First, it concerns the nature of the reactive oxygen species (ROS), which could be responsible for the antitumour effect of this therapy.

The effectiveness of HPD–PDT depends largely on the presence of oxygen [7], and it was reported [8] that $^1O_2$ is the main damaging agent in PDT. However, some in vitro [9–11] and in vivo studies [12–15] strongly suggest that other ROS, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{-\bullet}$), and hydroxyl ($OH^\bullet$) radicals, could also be involved in the HPD–PDT-induced tumour eradication. These oxidizers, especially $OH^\bullet$, can react with most biological molecules, including unsaturated lipids, DNA, and proteins, and cause cell death. It is important to note that the reactivity of $OH^\bullet$ towards the main cellular

constituents (such as lipids and proteins) exceeds considerably that for $^1O_2$. In the previous study [9], we established that photoexcited molecules of HPD can oxidize cellular proteins with the formation of semistable protein peroxides (PPO). The finding has important consequences for more precisely understanding the mechanism of the tumour ablating effect of HPD–PDT; currently, PPO are regarded as a new form of ROS, since decomposition of such peroxides has been demonstrated [16, 17] to yield reactive species, including oxygen- and carbon-centred radicals. These radicals can inactivate cellular enzymes [18], damage lipids and DNA [19], and could participate in the HPD–PDT induced killing of tumour cells [9]. At the same time, an analysis of the available literature showed that previous studies concerning the mechanism of H_2O_2, oxygen radicals, and PPO formation in tumour cells subjected to HPD–PDT are still fragmentary and inconclusive. For instance, it was reported [11] that photosensitization of transformed cells by HPD leads to the production of $OH^{\bullet}$; however, in this work the mechanism of this radical formation has remained to be elucidated.

The generation of H_2O_2, $O_2^{-\bullet}$, and $OH^{\bullet}$ during PDT could be explained in two ways. The first might involve direct electron transfer from photoexcited molecules of HPD to oxygen to give $O_2^{-\bullet}$ that after dismutation is converted into H_2O_2, a precursor of $OH^{\bullet}$. The other way could be associated with HPD-photosensitized oxidation of some biomolecules, both via type 1 and 2 reactions. Some data from the literature [20–22] suggest that photoexcited HPD itself, i.e. in the absence of photooxidizable biomolecules, has poor ability to produce H_2O_2, $O_2^{-\bullet}$, and $OH^{\bullet}$. We revealed [9] that in tumour cells treated with HPD–PDT the formation of these ROS could be explained via photooxidative transformations of their proteins. Besides, we showed that in tumour cells subjected to HPD–PDT part of the formed H_2O_2 and $O_2^{-\bullet}$ is transformed (by means of the Fenton-like reactions) into $OH^{\bullet}$ that reacts at, or close to, a diffusion-controlled rate with almost all biological molecules. At the same time, cells contain other molecules whose photooxidation could lead to the formation of H_2O_2 and $O_2^{-\bullet}$. In the present work, we tested the possibility that upon PDT the generation of H_2O_2 and $O_2^{-\bullet}$ in tumour cells could be mediated via HPD-photosensitized oxidation of the reduced forms of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH). Earlier, we discovered that in tumour cells subjected to HPD–PDT their proteins undergo photooxidative transformations with the formation of not only H_2O_2 but also PPO [9]. Moreover, our experiments showed that in the cells $^1O_2$ is involved in the generation of these peroxide compounds. Nevertheless, the nature of the amino acids whose oxidation by photoactivated molecules of HPD could lead to the formation of H_2O_2 and PPO remains obscure.

As known, mammalian cells, including transformed ones, are capable of producing marked amounts of H_2O_2, $O_2^{-\bullet}$, and $OH^{\bullet}$ [23]. Our prior studies showed that photosensitization of tumour cells by HPD strongly enhances their ability to generate of $O_2^{-\bullet}$ [9]. However, until now the mechanism of the phenomenon remains unclear. At present, mitochondria are considered as one of

the main sources of H_2O_2 and oxygen radicals; it has been estimated that up to 2% of the total oxygen consumed by the mitochondrial electron transport chain undergoes one electron reduction to generate $O_2^{-\bullet}$ and subsequently other ROS, such as H_2O_2 and $OH^{\bullet}$ [24]. In this relation, we assumed that upon PDT with HPD a damage of mitochondria may cause a rise in the production of $O_2^{-\bullet}$ by tumour cells. However, this hypothesis needs experimental checking.

In mammals, one of the major sources of ROS is xanthine oxidoreductase (XOR), which catalyses the last two reactions of purine catabolism by oxidizing hypoxanthine to xanthine and further to uric acid. This enzyme exists in two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XOD). They differ in that XDH utilizes NAD^+ preferentially as the electron acceptor, whereas XOD transfers reducing equivalents to molecular O_2. In the latter reaction, ROS, such as $O_2^{-\bullet}$ and H_2O_2, are formed [25]. As a result of its ability to generate ROS, XOD has received considerable attention as a pathological cause of ischemia-reperfusion injury [25], tissue damage associated with the inflammatory response [26], and cutaneous photosensitivity to haematoporphyrins [13]. Moreover, recent studies showed that photosensitization of tumour cells by HPD causes a very rapid and strong increase in the activity of XOD and that ROS generated by the enzyme could be involved in the antitumour effect of this photochemotherapy. Namely, Korbelik and co-workers [15], using mice with subcutaneously transplanted FsaR fibrosarcoma, found that photosensitization of the tumour by Photofrin induced a 5-fold increase in the activity of XOD. They concluded that the event could play an important role in the tumoricidal effect of PDT, since administration of oxypurinol (an inhibitor of XOD) reduced the tumour response to this phototherapy. However, in that article the causal relationship between the PDT-induced activation of XOD and formation of $O_2^{-\bullet}$ was not explored. We, in turn, revealed that in vitro the photodynamic treatment of Ehrlich ascites carcinoma (EAC) cells by HPD caused a considerable increase in the intracellular content of $O_2^{-\bullet}$, which was associated with a powerful (almost 3-fold) elevation in the activity of XOD. In the present work, using allopurinol (AP) as an effective and specific inhibitor of XOD [27], we estimated the amount of $O_2^{-\bullet}$ as well as the significance of ROS generated by XOD in the phototoxic action of HPD against tumour cells.

Clinical trials showed that during a standard regimen of PDT a considerable (5–13 °C) increase in the temperature of tumour tissues can take place [28]. Studies on various experimental tumours clearly demonstrated that these thermal effects associated with photoirradiation may play an important role in the total cytotoxic effect of HPD–PDT and may potentiate (in a synergistic manner) the porphyrin-photoinduced destruction of tumour cells [29–31]. However, until now the mechanism of the synergism has been studied insufficiently, though these observations led to the development of more effective treatment regimens in which HPD–PDT is combined with a localized laser- or microwave-induced hyperthermia immediately before or simultaneously with this phototherapy [30]. It was suggested that in PDT with HPD the hyperthermia produced by a laser

irradiation could improve the tumour response to the therapy via inhibiting the repair of photodynamically induced injuries [32], an increase in the reactivity of the formed $^1O_2$ [33], an inactivation of antioxidant defences of tumour cells [34], as well as by improvement of the oxygenation status of tumours [35]. At the same time, in a previous study [36] we found that the potentiating effect of heat shock on the antitumour efficiency of HPD–PDT could be largely explained by the stimulation of H_2O_2, $O_2^{-\bullet}$, and $OH^\bullet$ formation. Our investigations showed that the stimulatory effect of heating on the generation of these ROS may be attributed to an increase in the formation of a chlorin-type photoproduct, acting as a PS. However, there are other pathways that could mediate the enhancing effect of heat stress on the generation of H_2O_2 and oxygen radicals in tumour cells subjected to HPD–PDT. It was reported [37] that XDH can be reversibly converted to XOD by heating. In this connection, we supposed that heat shock in the course of PDT could promote processes causing the transformation of XDH to its oxidase form, and thereby the generation of $O_2^{-\bullet}$ in tumour cells. However, this assumption requires experimental checking.

Thus, the main aim of this work was to enrich the existing knowledge on the mechanism of H_2O_2, $O_2^{-\bullet}$, and PPO formation in tumour cells upon photo-activation of HPD. Also, we performed a study to clarify the mechanism of the stimulatory effect of heat stress on the generation of $O_2^{-\bullet}$ (a precursor of H_2O_2 and very cytotoxic $OH^\bullet$) in tumour cells subjected to PDT with HPD. This information is needed for a more precise understanding of the mechanism of the potentiating effect of heating, associated with the absorption of optical radiation, on the antitumour efficiency of PDT.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA, fatty acids free), H_2O_2 (30%, w/v), Cu/Zn superoxide dismutase (SOD; from bovine erythrocytes, 3300 units/mg protein), catalase (CAT; from bovine liver, thymol free, 16 700 units/mg protein), and all other chemicals (of analytical grade or better) were purchased from Sigma, St. Louis, MO, USA. HPD was prepared from haematoporphyrin dihydrochloride as described in [38].

Animals and cells

White, mongrel, three-month-old female mice obtained from the National Institute for Health Development (Tallinn, Estonia) were used in the experiments. The animals were fed ad libitum on standard pellets and had permanent access to water. The EAC cells obtained from the National Institute of Chemical Physics and Biophysics (Tallinn, Estonia) were maintained by intraperitoneal transplantation of 0.2 mL ascites fluid (ca 2.5×10^7 cells) from mouse to mouse every 7 days.

Light source

A voltage regulated 1 kW xenon arc lamp equipped with a focusing optical system and glass filters to deliver the light at 630 nm (range 590–830 nm) served as the radiation source in all the experiments; the infrared radiation was removed by a 4 cm water filter. The flux of the light was focused as a spot (2.54 cm^2) and directed on the front face of a quartz cuvette containing EAC cell suspension or HPD solutions. The intensity of the emitted light at 630 nm was always 260 mW with a power density of 102 mW/cm^2, as measured by an IMO-2N radiometer (Russian Federation).

Preparation of cell suspensions, the irradiation conditions, and cytotoxicity assay

Six- to seven-day-old EAC cells were withdrawn from the sacrificed animals, washed twice, and resuspended in phosphate-buffered saline (PBS) containing 154 mM NaCl, 6.2 mM KCl, 5.55 mM glucose, and 10 mM sodium phosphate buffer (pH 7.3). The viability of the cells was about 95–98%. Further, EAC cells were loaded with HPD (at 20 μg/mL for 25 min) exactly as described in our previous work [9] and kept in an ice bath until use. For the simultaneous thermal and PDT treatment, the cells loaded with HPD were resuspended in preheated PBS at a concentration of 5×10^6 cells/mL. An 8 mL sample of the cell suspension was quickly transferred into a 2×2 cm quartz cuvette and a microstirring magnet was added. The cuvette with the cells was then placed in a thermostatted (by circulating water) holder and illuminated in air with stirring at different temperatures, which were maintained within the error limits of $\pm 0.5\,^{\circ}C$. The time interval between the introduction of the cells into the preheated PBS and the beginning of light exposure was 5 min. Cytotoxicity was determined immediately after PDT by the trypan blue (TB) exclusion assay as described formerly [36].

Assessment of cell respiration

The respiration of EAC cells (1×10^7 cells/mL in air-saturated PBS) was estimated by measuring the rate of O_2 consumption by the cells using a Clark-type oxygen electrode. These measurements were performed in a water-thermostatted incubation chamber under continuous magnetic stirring at a required temperature.

Assay for $O_2^{-\bullet}$, H_2O_2, and PPO generation

The formation of $O_2^{-\bullet}$ in the control and HPD–PDT-treated EAC cells was estimated by the nitro blue tetrazolium (NBT) method [39] essentially as described previously [9]; the reduction of NBT by $O_2^{-\bullet}$ leads to the formation of a

blue-coloured formazan, which was quantified spectrophotometrically. Briefly, HPD-loaded EAC cells (5×10^6 cells/mL in PBS) were incubated in the dark or irradiated at various temperatures in the presence of 0.5 mM NBT, and the amount of the formed formazan was calculated by measuring the absorbance of cell lysates at 560 nm. The final results were expressed as nmoles NBT formazan per 1×10^6 cells. The ability of photoexcited HPD to generate $O_2^{-\bullet}$ was also examined in an aqueous buffer. In these experiments, the formation of $O_2^{-\bullet}$ was evaluated by monitoring the photosensitized reduction of NBT (see above). Unless otherwise indicated, the reaction mixtures were 10 μg/mL in HPD and 0.5 mM in NBT dissolved in PBS. The photoirradiation conditions were the same as for HPD-loaded EAC cells.

Cellular levels of H_2O_2 and PPO were determined using a FOX assay as described earlier [40, 41] with slight modifications. In this assay, these peroxides were reacted with an excess of Fe^{2+} at a low pH in the presence of the dye xylenol orange (XO) and the amount of Fe^{3+} generated was measured spectrophotometrically as the ferric–XO complex absorbs light in the visible range. Since H_2O_2 can easily diffuse across cellular membranes, the formation of H_2O_2 during photosensitization of EAC cells with HPD was estimated by the measurement of its concentration in cell-free supernatants. Immediately after light exposure, aliquots (1.0 mL) of the cell suspension (5×10^6 cells/mL) were placed into pre-cooled (on melting ice) plastic test tubes. The cells were then precipitated on an Eppendorf table centrifuge at maximal speed (8500 *g*) for 4 min and the concentration of H_2O_2 in the supernatants was determined by the FOX assay [40]. Namely, 0.9 mL of the supernatant was mixed with 0.9 mL of 50 mM H_2SO_4 and after the addition of 200 μL of a Fe–XO assay solution containing 2.5 mM $FeSO_4$ and 1 mM XO (in 25 mM H_2SO_4) the samples were vortexed and incubated in the dark at room temperature for 30 min. After the incubation, their absorbance at 560 nm was measured towards a blank probe. The peroxide concentrations were determined from a calibration curve that was constructed by measuring the absorbance of diluted samples of commercial H_2O_2 with known concentrations.

For the determination of PPO in EAC cells subjected to HPD–PDT, the cell lipids and proteins were separated by precipitating a known number of cells (ca 7×10^6) with 10% trichloroacetic acid (TCA) followed by centrifugation and a second TCA wash. In order to remove lipid peroxides, the final TCA precipitate was additionally washed with 2.5 mL of hexane/isopropyl alcohol (3 : 2, v/v) containing 0.02% butylated hydroxytoluene. The washed cell precipitates were then dried under a nitrogen flow and resuspended in 1.8 mL of 25 mM H_2SO_4. After the addition of the Fe–XO assay solution to the samples, protein hydroperoxides were assayed exactly as described for H_2O_2. The concentrations of PPO were calculated using the molar absorption coefficient of 35 500 M^{-1} cm^{-1} [41].

Determination of the intracellular levels of NAD(P)H, histidine, tryptophan, tyrosine, and protein-bound sylphydryl groups

Since NADH and NADPH are mainly responsible for the fluorescence band at 470 nm in normal as well as tumour cells [42], the content of NAD(P)H in EAC cells was estimated fluorometrically. Namely, during HPD–PDT the fluorescence of the cell suspension (5×10^6 cells/mL in PBS) at 470 nm (excitation at 340 nm) was measured in a 10×10 mm quartz cuvette under magnetic stirring with 5 nm bandwidths for excitation and detection. In the work, all fluorescence measurements were performed by a Hitachi 650-60 fluorescence spectrophotometer (Japan).

The tryptophan (Trp) content of cellular proteins was measured according to the method of Spies & Chambers [43], histidine (His) and tyrosine (Tyr) according to Sokolovsky & Vallee [44], whereas protein-bound sulphydryl groups (PrSH) were assayed by the Ellman method with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) exactly as described in [45].

HPD-photosensitized oxidation of Trp, Tyr, His, and BSA in aqueous solution

In these experiments, an 8 mL sample of a 10 μg/mL HPD solution (containing BSA or amino acids dissolved in PBS) was placed into a 2×2 cm quartz cuvette and irradiated in air under magnetic stirring at 30 °C with red light at 630 nm. After the cessation of photolysis, the absorbance (differential spectrum) of Trp was recorded to determine the rate of Trp degradation in water solution, while the concentrations of Tyr and His were quantified exactly as described in the previous section. Under photoexcitation of HPD, the levels of His, Trp, and Tyr in BSA were also measured according to the above-mentioned procedures for cell proteins, whereas the content of SH-groups was determined by the DTNB method as described in [46]. During the HPD-sensitized photooxidation of amino acids and BSA, the yields of peroxide compounds were quantified by the FOX assay [40] using H_2O_2 as the standard. The content of amino acid peroxides (AAP) as well as PPO was determined after the incubation of photoirradiated samples with CAT (400 units/mL) for 15 min at room temperature. Concentrations of the formed H_2O_2 were calculated by subtracting the value obtained for AAP or PPO from that for total peroxides.

Quantification of XOD and total XOR activities in EAC cells

The activities of XOD and total XOR were measured in cell lysates at 37 °C by a very sensitive fluorometric assay [47] with minor modifications. At different times after photodynamic treatment, EAC cells (ca 2×10^6) were washed and resuspended in 0.5 mL of PBS. The washed cells were sonicated on ice for 5 s using a Torbeo cell disrupter (USA) at average power. Further, 0.4 mL of lysed cell suspension was added to 1.07 mL of preheated PBS. The reaction was

initiated by addition of 30 μL of 0.5 mM pterine, and the change in fluorescence at 390 nm (excitation at 345 nm) with time was recorded on a spectrophotometer under magnetic stirring. The activity of XOD was determined by the rate of change in fluorescence with time in the presence of pterine alone. Methylene blue (15 μL of 1 mM) was then added, as an electron acceptor, and the change in fluorescence was again recorded to measure total XOR activity. The reaction was then stopped by addition of AP to a final concentration of 10 μM. The fluorescence at 390 nm was measured before and after the addition of 15 μL of 10 μM isoxanthopterin to the assay mixture as an internal standard. The activities of XOD and XOR were expressed as nmoles isoxanthopterin formed/min per mg cell protein. The total cell proteins were determined by the well-known procedure with fluorescamine, using BSA as the standard.

Statistics

Results were analysed statistically by the Student's *t*-test. Values of $P < 0.05$ were considered statistically significant. Data in the text, tables, and figures are presented as mean ± standard error (SE) of at least three separate experiments.

RESULTS AND DISCUSSION

On the mechanism of the generation of ROS in tumour cells upon PDT with HPD

In these studies, HPD-loaded EAC cells were suspended in PBS and then illuminated in air with red light at 630 nm for 15 min (the temperature was 30 °C). Figure 1 shows that under photoexcitation of HPD, the kinetics of the EAC cells inactivation was of S type; after a lag (ca 5 min), an avalanche like increase in the number of dead cells was observed. In this work, the light dose at which 50% of the cells were stained by TB (LD_{50}) was utilized as a criterion to evaluate the efficiency of HPD–PDT-induced cytotoxicity. However, irradiation of EAC cells in the absence of HPD as well as their incubation with the PS in the dark did not cause any noticeable increase in the number of dead cells.

We found that photosensitization of EAC cells by HPD resulted in a drastic injury of their mitochondria; namely, after 14 min irradiation a considerable (>80%) decrease in the rate of O_2 consumption by the cells was registered (Fig. 1b). It was also established that irradiation of HPD-loaded EAC cells caused a considerable (ca 30%) fall in the intracellular content of NAD(P)H as well as serious oxidative damages to their proteins, which was expressed as a substantial modification of the amino acid composition of cell proteins; namely, at light doses causing an irreversible inactivation of about 95% of the cells, the proportion of the photooxidized amino acid residues was determined as 34% for His, 20% for PrSH, 16% for Trp, and 11% for Tyr (Fig. 1c). By contrast, a prolonged incubation of EAC cells with the PS in the dark or irradiation of the

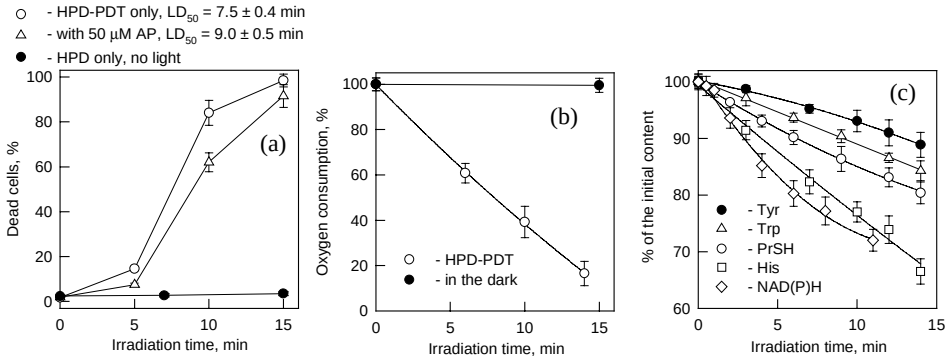

Fig. 1. Kinetics of photoinactivation of EAC cells (5×10^6 cells/mL in PBS) (a) at 30 °C and the effects of HPD-induced photosensitization on their respiratory activity (b), the intracellular content of tyrosine (Tyr), tryptophan (Trp), histidine (His), protein-bound SH groups (PrSH), and NAD(P)H (c). The initial levels of Tyr, Trp, His, and PrSH in the non-irradiated cells were determined as (per 1×10^6 cells): 33.9 ± 0.4 nmoles for Tyr, 20.4 ± 0.2 nmoles for Trp, 34.1 ± 0.3 nmoles for His, and 17.1 ± 0.7 nmoles for PrSH. The rate of O_2 consumption by intact EAC cells was measured (at 37 ºC) as 12.1 ± 0.2 nmoles O_2/min per 1×10^7 cells. AP, allopurinol. LD_{50}, the light dose at which 50% of the cells were stained by TB. Bars are SE.

cells not exposed to HPD did not induce similar changes. Thus, our experiments showed that photosensitization of EAC cells with HPD induced a potent oxidative damage to proteins, which was associated with an inactivation of their mitochondria, which, in turn, well correlated with the increase in the number of dead cells.

It was found that irradiation of HPD-loaded EAC cells led to the generation of considerable amounts of H_2O_2, $O_2^{-\bullet}$, and PPO (Fig. 2). Furthermore, in a prior study [9], we clearly demonstrated that these ROS are involved in the phototoxic action of HPD against the cells. Our observation that the $O_2^{-\bullet}$ and H_2O_2 formation is associated with the HPD–PDT treatment is supported by the data of other researchers. For example, Athar et al. [12] studied the mechanism of HPD-mediated cutaneous photosensitization (a main drawback of PDT with the PS) in mice and revealed that both inhibitors of SOD (a scavenger of $O_2^{-\bullet}$) and CAT (a scavenger of H_2O_2) augmented the skin photosensitivity. Korbelik et al. [14] found that intravenous SOD administration immediately after light exposure decreases the cure rate of HPD–PDT-treated mouse FsaR and EMT6 tumours. Our further work was aimed at clarifying the mechanism of $O_2^{-\bullet}$, H_2O_2, and PPO generation in tumour cells subjected to PDT with HPD.

Using NBT as an indicator substance, we revealed that photosensitization of EAC cells by HPD caused a very rapid and considerable increase in the intracellular content of $O_2^{-\bullet}$, which was strongly inhibited by exogenously added Cu/Zn–SOD, a trap of $O_2^{-\bullet}$ (Fig. 2a). By contrast, only negligible levels of $O_2^{-\bullet}$ were detected in EAC cells after their irradiation in the absence of HPD, during incubation of HPD-loaded cells in the dark, or upon illumination of HPD in PBS

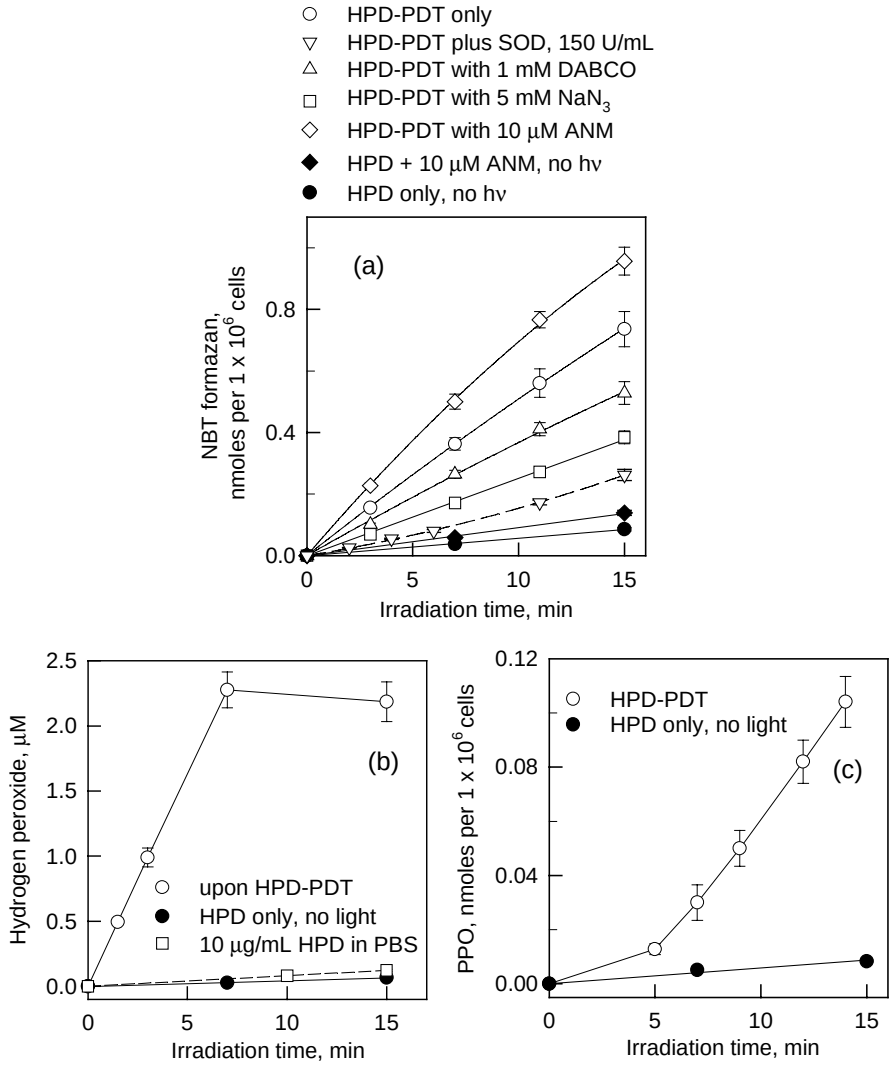

Fig. 2. The generation of $O_2^{-\bullet}$ (a), H_2O_2 (b), and protein peroxides (c) during photosensitization of EAC cells (5×10^6 cells/mL in PBS) with HPD or their incubation in the dark at 30 °C. ANM, antimycin A. DABCO, 1,4-diazabicyclo[2.2.2]octane. NBT, nitro blue tetrazolium. PPO, protein peroxides. SOD is erythrocyte Cu/Zn-SOD. Bars are SE.

at a concentration close to that in EAC cells (10 μg/mL). The obtained results suggest that in tumour cells subjected to HPD–PDT, the formation of $O_2^{-\bullet}$ may be ascribed partly to $^1O_2$-mediated reactions; in fact (Fig. 2a), under the PDT the yields of $O_2^{-\bullet}$ generation in EAC cells were markedly decreased in the presence of NaN_3 and 1,4-diazabicyclo[2.2.2]octane, well-known quenchers of $^1O_2$. Using the FOX assay, we revealed that irradiation of EAC cells loaded with HPD led to the formation of significant (>2 μM) amounts of H_2O_2 (Fig. 2b). Like $O_2^{-\bullet}$, the

levels of H_2O_2 strongly increased within the first 6 min of irradiation and attained a plateau after 10–12 min of light exposure. Only minor levels of H_2O_2 were detected after irradiation of EAC cells in the absence of HPD, incubation of HPD-loaded cells in the dark, or at illumination of the PS in cell-free PBS. Thus, our experiments clearly demonstrated that photoexcited HPD itself has poor ability to produce H_2O_2 and $O_2^{-\bullet}$; only trace amounts of these ROS were detected after illumination of water solutions of the PS. The finding is consistent with the data from other laboratories [20–22].

Our data suggest that the formation of $O_2^{-\bullet}$ (a precursor of H_2O_2) in tumour cells subjected to PDT could be mediated via the HPD-photosensitized damage to their mitochondria. In fact (Figs 1b and 2a), we found that in the EAC cells treated with HPD–PDT the impairment of the mitochondrial function was directly related to the generation of $O_2^{-\bullet}$ and that the yield of $O_2^{-\bullet}$ formation was substantially (by 30%) enhanced upon illumination of these cells in the presence of 10 μM antimycin A (ANM), an inhibitor of the Complex III at the site of ubiquinol–cytochrome *c* reductase in the respiratory chain of mitochondria [39]. It is important to note that the stimulatory effect of ANM on the generation $O_2^{-\bullet}$ was considerably lesser in comparison with control cells, which were incubated in the dark at 30 °C. Our findings on the responsibility of mitochondria for an increased generation of $O_2^{-\bullet}$ in HPD–PDT-treated tumour cells are consistent with the data from other laboratories; namely, Salet et al. [48] reported that photosensitization of isolated mitochondria by HPD greatly increases their capability to produce $O_2^{-\bullet}$. However, in this work the mechanism of the phenomenon was not explored. We assume that an elevated production of $O_2^{-\bullet}$ by mitochondria of HPD–PDT-treated cells could induce by leakage of cytochrome *c* into the cytosol; this is a well documented event in PDT of tumours [49], which, as believed, is the main cause of photodynamically-induced suppression of cell respiration and apoptosis. The release of cytochrome *c* may cause a burst in the generation of $O_2^{-\bullet}$ due to an increase in the level of the unstable ubisemiquinone [50]; its auto-oxidation leads, as known, to the generation of $O_2^{-\bullet}$. However, this hypothesis will be checked in our further studies.

In order to clarify the significance of ROS generated by XOD in the tumouricidal effect of PDT, the effect of AP (a specific inhibitor of XOD [27]) on the formation of $O_2^{-\bullet}$ as well as the phototoxic action of HPD against EAC cells was examined in vitro. We found that photosensitization of EAC cells by HPD led to a strong (>2-fold) increase in the activity of XOD, which correlated well with the generation of $O_2^{-\bullet}$ in the PDT-treated cells (Figs 8a and 2a, respectively). Control experiments showed that the initial activity of XOD in EAC cells not exposed to HPD was relatively low (11.7 ± 0.7 pmoles isoxanthopterin formed/min per mg cell protein) and altered negligibly after exposure of the cells to HPD, upon incubation of HPD-loaded cells in the dark, as well as after illumination of cells in the absence of the PS. Our results strongly suggest that in tumour cells subjected to HPD–PDT, a substantial part of the formed $O_2^{-\bullet}$ may be generated by XOD. Indeed (Fig. 3), the production of $O_2^{-\bullet}$ in

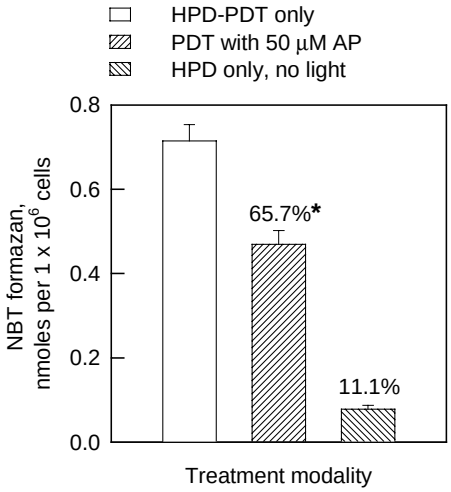

Fig. 3. Influence of allopurinol (AP) on the generation of $O_2^{-\bullet}$ in EAC cells (5×10^6 cells/mL in PBS) subjected to HPD–PDT. The effect of AP was tested after 14 min photoirradiation of HPD-loaded EAC cells without or in the presence of this compound; the temperature was 30 °C. The intracellular levels of $O_2^{-\bullet}$ were assayed by the NBT method. *****, % of the value upon HPD–PDT. Bars are SE.

HPD-loaded EAC cells was notably (by ca 35%) decreased after their irradiation in the presence of 50 μM AP; at this concentration, AP completely blocked the HPD–PDT-induced rise in the activity of XOD in the cells. Moreover, AP at the concentration studied (50 μM) induced a marked (ca 20%) decrease in the rate of HPD-sensitized photoinactivation of EAC cells (Fig. 1a). Based on the data, we concluded that the ROS generated by XOD must play an important role in the antitumour effect of PDT with HPD.

In the further experiments, we provided unambiguous evidence that in cell proteins the HPD-photosensitized oxidation of aromatic amino acids may be responsible for the generation of peroxide compounds. In fact, upon HPD–PDT the generation of H_2O_2 and PPO in EAC cells was associated with a pronounced decrease in the content of Trp, Tyr, and His residues (Figs 1 and 2). At the same time, similar chemical events were registered during HPD-photosensitized oxidation of BSA; in the model protein the photoinduced oxidation of Trp, Tyr, and His was also associated with the formation of large amounts of H_2O_2 and PPO (Fig. 4). Furthermore, we found that in an aqueous buffer the HPD-photosensitized oxidation of His, Tyr, and Trp resulted in the formation of significant amounts of H_2O_2 and AAP (Fig. 5). The yields of HPD-photoinduced generation of peroxide compounds vary greatly depending on the chosen amino acid; namely, upon oxidation of Tyr the yields of H_2O_2 generation exceed considerably those for Trp and His. However, in the case of Trp, the yield of AAP was much higher in comparison with His and Tyr (Table 1). Our experiments showed that PPO are not the major products of photooxidative trans-

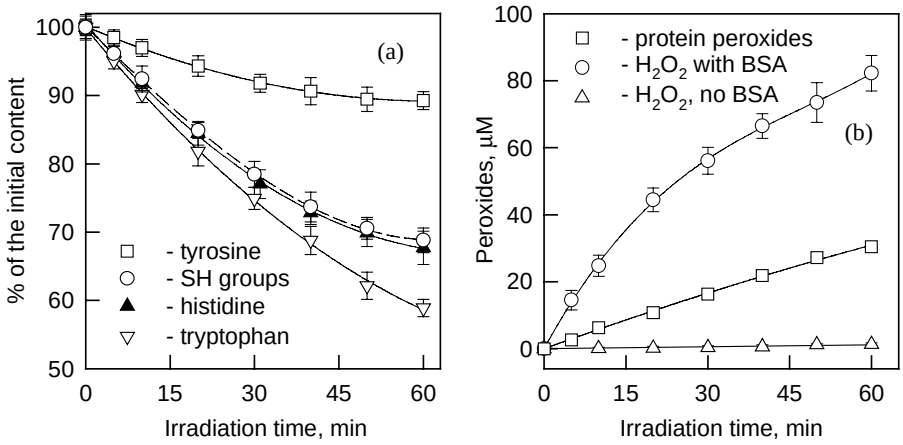

Fig. 4. The kinetics of 10 μg/mL HPD-photosensitized (λ_{max} = 630 nm, 102 mW/cm^2) degradation of bovine serum albumin (BSA, 10 mg/mL in PBS) at 30 °C (a) and the generation of peroxide compounds during HPD-photoinduced oxidation of BSA (b). The initial content of photooxidizable amino acid residues in BSA was determined as (per mg protein): 159 ± 6 nmoles for histidine, 184 ± 7 nmoles for tyrosine, 45 ± 2 nmoles for tryptophan, and 84 ± 4 nmoles for SH groups. Bars are SE.

formations of proteins. Namely, during HPD-photosensitized oxidation of BSA, the yield of H_2O_2 generation greatly exceeded that for PPO; the molar ratio H_2O_2/protein-bound peroxides was roughly constant, approximately 3.0 : 1 at all illumination times (Fig. 4). The same ratio of H_2O_2/PPO was monitored upon photosensitization of EAC cells with HPD; these data suggest that the contribution of H_2O_2 in the damaging action of HPD–PDT against EAC cells prevailed over that from PPO.

It was reported [21] that HPD and light in the presence of cysteine may convert molecular O_2 to H_2O_2 and $O_2^{-\bullet}$. As the photosensitization of EAC cells by HPD led to a strong fall in the level of PrSH (Fig. 1c), we believe that upon PDT the formation of H_2O_2 and $O_2^{-\bullet}$ in tumour cells could be induced via oxidation of cysteine residues in cell proteins. This is supported by the model experiments with BSA; in the protein the HPD-photosensitized oxidation of SH groups was associated with the generation of H_2O_2 (Fig. 4).

Our studies point to the possibility that the generation of H_2O_2 and $O_2^{-\bullet}$ in tumour cells subjected to PDT could be mediated not only by the HPD-photosensitized oxidation of proteins, but also by NAD(P)H; indeed, upon irradiation of HPD-loaded EAC cells simultaneously with the formation of these ROS, a clearly expressed (ca 30%) lowering in the intracellular content of these nucleotides was observed (Fig. 1c). Our findings are supported by the data of other researchers [21, 51], who discovered that HPD-catalysed photooxidation of NAD(P)H in aqueous solution leads to the generation of H_2O_2 and $O_2^{-\bullet}$.

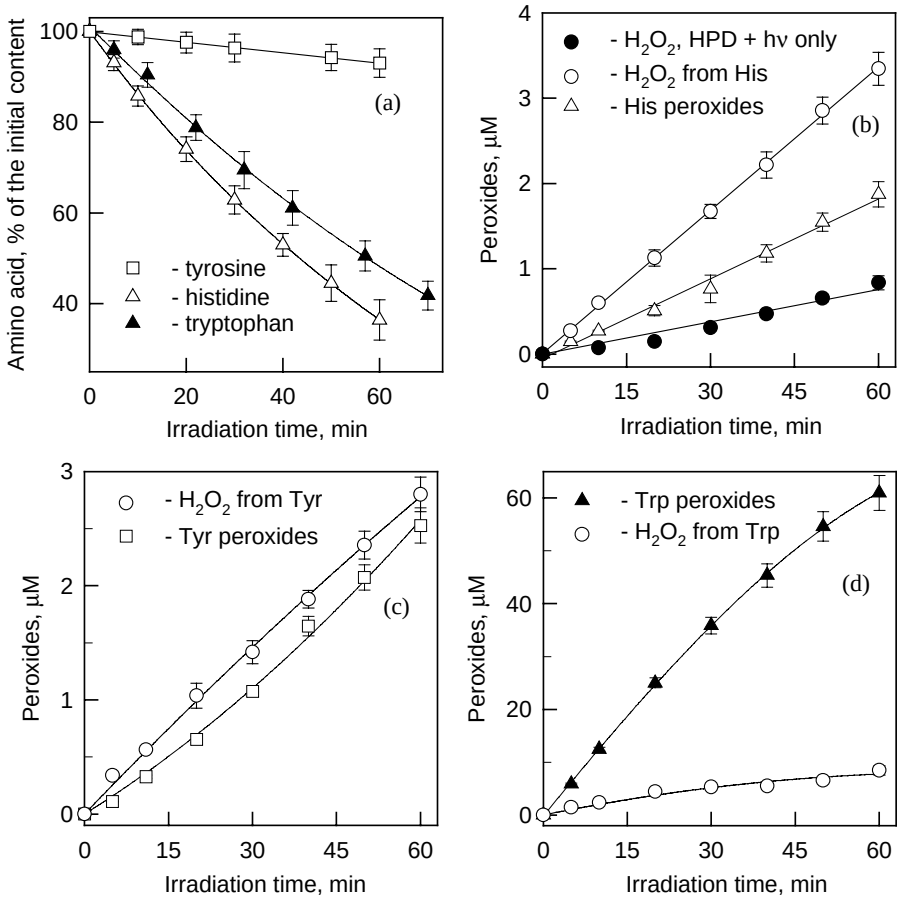

Fig. 5. The kinetics of 10 μg/mL HPD-photosensitized (λ_{max} = 630 nm, 102 mW/cm^2) oxidation of 0.2 mM histidine (His), tyrosine (Tyr), and tryptophan (Trp) (a) in PBS and the generation of peroxide compounds during photodestruction of His (b), Tyr (c), and Trp (d). The temperature was 30 °C. Bars are SE.

Table 1. The yields of peroxide compounds formation following 10 μg/mL HPD-photosensitized (λ_{max} = 630 nm, 102 mW/cm^2) oxidation of aromatic amino acids in an aqueous buffer at 30 °C

Amino acid[a]	Yield of amino acid peroxides, %[b]	Yield of H_2O_2 formation, %[b]
L-histidine	1.4	2.1
L-tyrosine	17.4	14.9
L-tryptophan	58.8	6.7

[a] – The reaction mixtures contained 0.2 mM histidine, tyrosine, or tryptophan dissolved in PBS.

[b] – The yields were calculated after 60 min light exposure.

Effect of hyperthermia on the response of tumour cells to HPD–PDT and generation of ROS

In these studies, HPD-loaded EAC cells were irradiated with red light at 630 nm or incubated in the dark at three different temperatures (30, 37, and 44 °C). The 30 °C group of cells was taken as the control, as there is increasing enthusiasm (due to excellent cosmetic results) for the use of porphyrin sensitizers, including HPD, in PDT of skin cancers having the surface temperature close to 30 °C. Experiments showed that rising the irradiation temperature from 30 to 44 °C substantially (by about 1.5-fold) increased the rate of HPD-photosensitized killing of EAC cells (Fig. 6a). However, incubation of the cells in the dark even at hyperthermic (44 °C) temperature did not induce any noticeable increase in the number of dead cells. It was also found that sub- (37 °C) and hyperthermic (44 °C) heating accelerated the HPD-photosensitized damage of cellular proteins; namely upon rising the temperature from 30 to 44 °C a marked (ca 1.7-fold) growth in the rate of PrSH photooxidation was monitored (Fig. 6b). However, heat shock itself did not induce similar changes in the composition of cell proteins. Thus, our data support the view [29–31, 52] that in laser photochemotherapy the mild hyperthermia (around 44 °C) produced by irradiation can enhance synergistically the HPD-photoinduced tumour eradication.

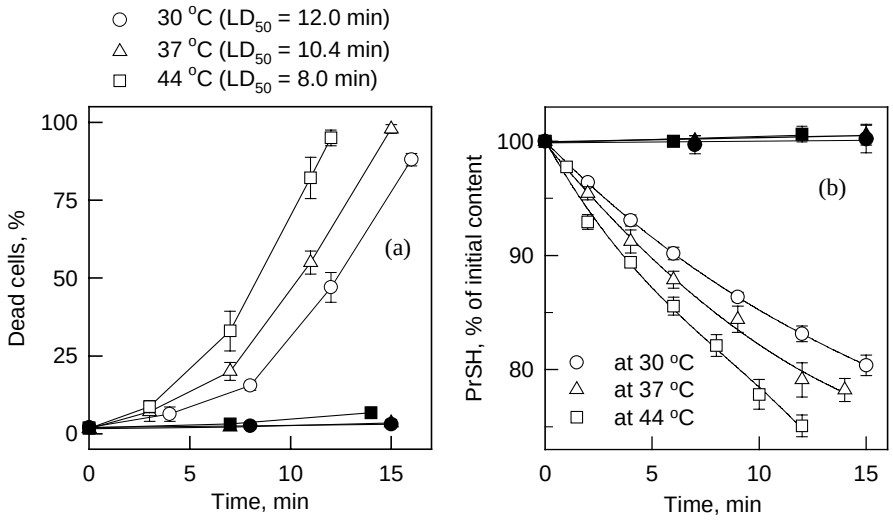

Fig. 6. Kinetics of inactivation of HPD-loaded EAC cells (5×10^6 cells/mL in PBS) (a) and the content of protein-bound sulphydryl groups (PrSH) in the cells (b) during their photoirradiation (open symbols) or incubation in the dark (filled symbols) at different temperatures. The initial content of PrSH in the non-irradiated EAC cells was determined as 17.1 ± 0.7 nmoles per 1×10^6 cells. LD_{50} is the light dose at which 50% of the cells were stained by TB. Bars are SE.

Heat stress during PDT could enhance the HPD-induced photooxidative damage to cellular constituents and, as a consequence, killing of tumour cells not only via an increase in the reactivity of $^1O_2$ [33], but also through the stimulation of other ROS formation. In fact (Fig. 7), we found that upon HPD–PDT a rise in the temperature from 30 to 44°C strongly (by ca 2.5-fold) enhanced the generation of $O_2^{-\bullet}$ in EAC cells, which correlated well with the increase in the rate of their photosensitized killing. In order to clarify the mechanism of a heat-catalysed increase in the formation of $O_2^{-\bullet}$, we examined the effect of temperature on the activity of XOD in HPD-loaded EAC cells upon their photoirradiation or incubation in the dark. In this work we established that in the cells subjected to the phototoxic action of HPD part (ca 30%) of the formed $O_2^{-\bullet}$ is produced by XOD, and that ROS generated by this enzyme are involved in cell death (Figs 1 and 3, respectively). Experiments showed that heating contributed to the PDT-induced rise in the activity of the ROS producing enzyme; namely, the 12 min treatment of EAC cells by HPD–PDT at the control (30°C) temperature caused an about 2-fold growth in the activity of XOD, whereas the same light exposure at 44°C led already to a 2.7-fold increase in the activity of the enzyme (Fig. 8a). What is the mechanism of the phenomenon? We believe that heat shock in the course of PDT could promote photochemical processes causing the transition of XDH to its oxidase form, since incubation of HPD-loaded EAC cells in the dark or irradiation of the cells not exposed to HPD even at a hyperthermic (44°C) temperature had no effect on the activity of XOD (Fig. 8a). The conversion of XDH to XOD may occur reversibly through oxidation of SH groups or irreversibly by limited proteolysis [25]. In a prior work [9], we discovered that in tumour cells treated with HPD–PDT an increase in the activity of XOD is induced by the conversion of XDH to its oxidase form, predominantly, via the oxidation of SH groups in XDH. At the same time,

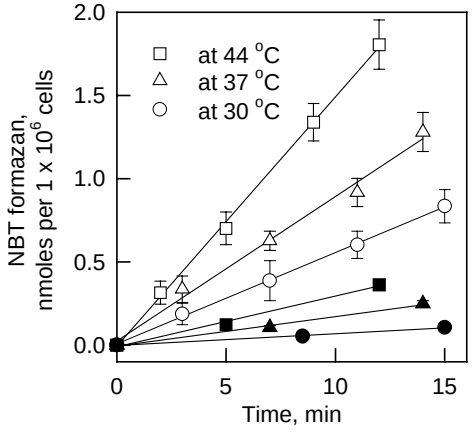

Fig. 7. The formation of $O_2^{-\bullet}$ in HPD-loaded EAC cells (5×10^6 cells/mL in PBS) during their photoirradiation (open symbols) or incubation in the dark (filled symbols) at different temperatures. NBT, nitro blue tetrazolium. Bars are SE.

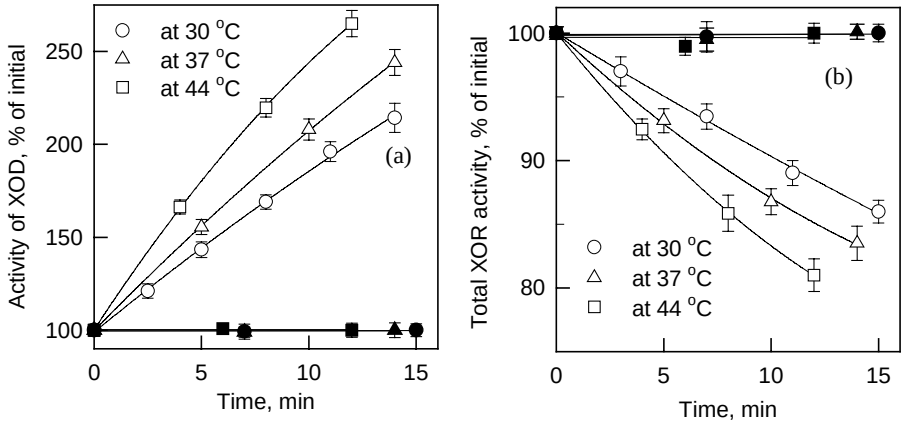

Fig. 8. Xanthine oxidase (XOD) (a) and total xanthine oxidoreductase (XOR) (b) activities in HPD-loaded EAC cells (5×10^6 cells/mL in PBS) during their photoirradiation (open symbols) or incubation in the dark (filled symbols) at different temperatures. The initial XOD and total XOR activities in the non-irradiated EAC cells were measured as 11.7 ± 0.7 and 36.4 ± 1.8 pmoles isoxanthopterin formed/min per mg cell protein, respectively. Bars are SE.

experiments on EAC cells showed that heating caused a strong increase in the rate of HPD-photosensitized oxidation of cysteine thiols in cell proteins (Fig. 6b). Hence, it could be assumed that upon HPD–PDT at elevated temperatures a rise in the activity of XOD in EAC cells was largely mediated by a heat-catalysed increase in the photooxidation of SH groups in XDH. Studies showed that in EAC cells the PDT-induced increase in the activity of XOD was associated with a decrease in the total (XDH + XOD) XOR activity and that the suppression of XOR activity enhanced upon heating the cells (Fig. 8b). This could be conditioned by photoinactivation of XDH. However, the fall in the total XOR activity even at a hyperthermic (44 °C) temperature was relatively small (not more than 18%), suggesting that in tumour cells XDH is persistent to HPD–PDT-induced inactivation.

Our investigations showed that heat stress itself promotes the generation of ROS in tumour cells. Indeed, upon incubation of EAC cells in the dark a rise in the temperature from 30 to 44 °C caused a considerable (over 2-fold) increase in the rate of $O_2^{-\bullet}$ production by the cells (Fig. 7). This finding is in good agreement with the observation of other researchers [53], who discovered that heating (from 37 to 43–45 °C) of non-transformed as well as tumour cells strongly enhances the generation of oxygen radicals in the cells. Until now, the precise location and mechanisms of increased formation of oxygen-derived free radicals upon heat stress have been studied insufficiently. As in tumour cells XOD is found to be involved in the generation of ROS (Fig. 3), we assumed that heating could elevate the activity of this enzyme in the cells and, thereby, the formation of $O_2^{-\bullet}$. In this connection, we examined the effect of heating on the activity of XOD in intact, not exposed to HPD–PDT, EAC cells. It was established that raising the

temperature of a medium from 30 to 44 °C induced more than a 2-fold increase in the activity of XOD in the cells; however, further heating from 44 to 60 °C resulted in the inactivation of this enzyme (Fig. 9). There are some indications that the mitochondrial electron transport chain could be also responsible for an increased production of $O_2^{-\bullet}$ and H_2O_2 in cells subjected to a thermal stress [54]. Our results are consistent with these data. In fact, we discovered that within the temperature range of 30–44 °C, the ability of EAC cells to produce $O_2^{-\bullet}$ is directly related to their respiratory activity (Fig. 10). Moreover, recently we found that

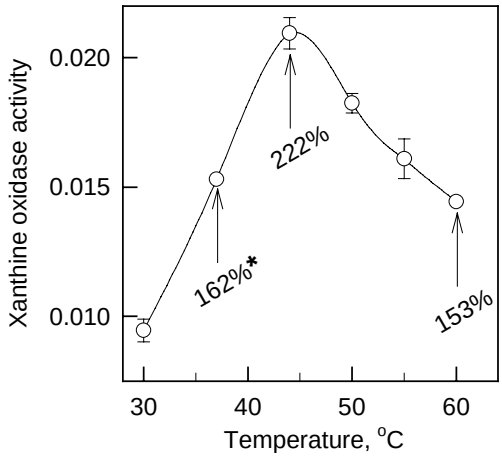

Fig. 9. Influence of temperature on the activity of xanthine oxidase in EAC cells. The enzyme activity is expressed as nmoles isoxanthopterin formed/min per mg cell protein. *****, % of the value obtained at 30 °C. Bars are SE.

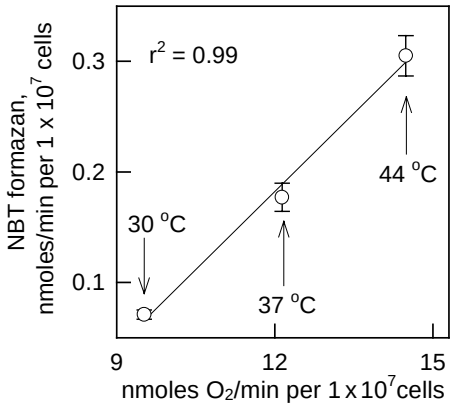

Fig. 10. The interrelationship between the rate of $O_2^{-\bullet}$ production by non-illuminated EAC cells and their respiratory activity at various temperatures. NBT, nitro blue tetrazolium. Bars are SE.

heating (from 30 to 44 °C) exerts a potent stimulating effect on the production of H_2O_2 in EAC cells, which correlated well with a rise in the rate of O_2 consumption by the cells [36]. Thus, taken together, our studies strongly suggest that XOD, along with the respiratory chain of mitochondria, may be responsible for an increased formation of $O_2^{-\bullet}$ and H_2O_2 in tumour cells at hyperthermic (up to 44–45 °C) temperatures. They, jointly with photochemically generated oxidants, could take part in the HPD–PDT-induced destruction of tumours.

CONCLUSIONS

Previous studies suggested that the predominant tumouricidal mechanism in HPD–PDT is through a type 2 photochemical reaction associated with the formation of $^1O_2$. At the same time, there are some indications that besides $^1O_2$ other active oxygen forms (such as $O_2^{-\bullet}$, $OH^{\bullet}$, H_2O_2, and PPO) could also participate in the tumour necrotizing effect of PDT with HPD. However, until now many aspects related with the mechanism of oxygen radicals as well as the generation of peroxide compounds in tumour cells subjected to the phototoxic action of HPD remained unclear. In this work, we discovered that the phototoxic action of HPD against tumour cells is associated with the formation of considerable amounts of $O_2^{-\bullet}$, H_2O_2, and PPO. Studies on the mechanism showed that photoexcited molecules of HPD itself are a poor source of H_2O_2 and oxygen radicals, and that in tumour cells subjected to PDT the generation of H_2O_2 and $O_2^{-\bullet}$ (precursors of $OH^{\bullet}$) could be largely explained by HPD-photosensitized oxidation of certain cellular constituents (NAD(P)H, proteins) as well as via an increase in the activity of XOD. Besides, we concluded that upon HPD–PDT a damage to mitochondria may be responsible for an increased formation of $O_2^{-\bullet}$ in target cells. Our experiments clearly demonstrated that ROS generated by XOD may play an important role in the antitumour effect of HPD–PDT. It was also established that in cell proteins the HPD-photosensitized oxidation of aromatic amino acids results in the generation of H_2O_2 and protein-bound peroxides.

One of the major findings of our studies is that upon HPD–PDT the hyperthermia produced by photoirradiation may enhance the PDT-induced oxidative stress and, as a result, its tumouricidal effect via a rise in the activity of XOD as well as the stimulation of $O_2^{-\bullet}$ production by the respiratory chain of mitochondria. Upon PDT the temperature of tumour lesions is commonly not controlled. In our opinion, a tight control of tumour surface temperature during HPD–PDT, especially when it is combined with simultaneous local hyperthermia, is needed to obtain the maximal value of tumour necrosis. Indeed, our results as well as some data from the literature suggest that severe hyperthermia (at temperatures >45 °C) could induce, contrary to mild hyperthermia (around 42–44 °C), a reduction in the antitumour efficiency of HPD–PDT; we found that heating (from 30 to 44 °C) of EAC cells caused a substantial (>2-fold) increase in the activity of XOD, whereas further elevation of temperature (up to 60 °C) led to

a fall in the activity of the ROS producing enzyme. Further, PDT with HPD is based on a photochemical reaction that is limited by the availability of molecular oxygen in the tumour tissues [7]. However, it was reported that severe hyperthermia may cause a drastic drop in tumour oxygen tension due to an almost complete cessation of nutritive blood flow [35].

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Hapniku reaktiivsete osakeste tekke mehhanismi uurimine kasvajarakkudes hematoporfüriini derivaadi fototoksilisel toimel: temperatuuri mõju

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Töö eesmärgiks on: 1) H_2O_2, superoksiidi ($O_2^{-\bullet}$) ja proteiinide peroksiidide (PPO) tekkemehhanismi uurimine kasvajarakkudes hematoporfüriini derivaadi (HPD) fotoaktivatsioonil; 2) termilise stressi stimuleeriva toime mehhanismi selgitamine $O_2^{-\bullet}$ (H_2O_2 ja väga aktiivsete hüdroksüülradikaalide eelkäija) tekkele kasvajarakkudes HPD manulusel fotodünaamilise teraapia (FDT) käigus. Eksperimentides kasutatud Ehrlichi astsiitse kartsinoomi (EAC) rakke on inkubeeritud HPD fosfaatpuhvris ja seejärel kiiritatud punase valgusega 630 nm. On leitud, et HPD fotoergastamine põhjustab NAD(P)H taseme languse rakkudes ja kahjustab oluliselt raku valke ja mitokondreid. Seejuures kaasneb nende nähtustega olulises koguses H_2O_2, $O_2^{-\bullet}$ ja PPO teke. HPD enda fotoergastamine annab vähesel hulgal H_2O_2 ja hapniku radikaale. H_2O_2 ja hapniku radikaalide teke rakkudes võib olla tingitud: (i) teatud raku koostisosade (NAD(P)H) fotooksüdatsioonist; (ii) ksantiini oksüdaasi (XOD) aktiivsuse kasvust; (iii) mitokondrite fotovigastamisest. HPD fotosensibiliseeritud aminohapete oksüdatsioon toimub läbi H_2O_2 ja PPO tekke. Autorite uurimuse kohaselt suurendab pehme hüpertermia (*ca* 44 °C) HPD kasvajavastast toimet tänu $O_2^{-\bullet}$ tekke stimuleerimisele; temperatuuri tõus 30–44 °C põhjustab olulise (*ca* 2,5-kordse) $O_2^{-\bullet}$ tekke suurenemise EAC-rakkude kiiritamisel ja see korreleerub rakkude HPD fotohävimise kiiruse suurenemisega. Uurimusest nähtub, et $O_2^{-\bullet}$ tekke intensiivistumine on tingitud soojendamise stimuleerivast mõjust XOD aktiivsusele ja radikaalide tekke stimulatsioonist mitokondrite hingamisahelas. Uurimusest tuleneb, et kõrge hüpertermia (üle 45 °C), erinevalt pehmest hüpertermiast, pärsib HPD-FDT efektiivsust, mis on tingitud XOD aktiivsuse supressioonist kasvajarakkudes.