Influence of heating on the activity of xanthine oxidase in tumor cells subjected to the phototoxic action of hematoporphyrin derivative

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The aim of this study was to clarify the mechanism of the stimulatory effect of heat stress on generation of superoxide radical ($O_2^{\bullet}$) in tumors subjected to photodynamic therapy (PDT) with hematoporphyrin derivative (HPD). For this purpose, the effect of heating on the activity of xanthine oxidase (XOD) in tumor cells upon their photosensitization with HPD was examined; this enzyme is participated in purine catabolism and has the ability to generate $O_2^{\bullet}$, a precursor of H_2O_2 and very cytotoxic hydroxyl radical. The study was carried out on Ehrlich ascites carcinoma (EAC) cells, which were loaded with HPD in a serum-free medium and then irradiated with red light (λ_{max} = 630 nm) at 3 different temperatures (30, 37 and 44 °C). In the cells, the activity of XOD was assayed fluorometrically, using pterine as the substrate, whereas the production of $O_2^{\bullet}$ by the nitro blue tetrazolium method. It was found that increasing of the temperature from 30 to 44 °C strongly (by ~ 2.5-fold) enhanced the generation of $O_2^{\bullet}$ in EAC cells that correlated well with an increase in the rate of their photosensitized killing. Experiments showed that the intensification of $O_2^{\bullet}$ formation could be mediated by the stimulatory effects of heating on the activity of XOD; namely, the 12 min treatment of EAC cells by HPD-PDT at a 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 this enzyme. However, incubation of EAC cells in the dark even at a hyperthermic (44 °C) temperature had no effect on their XOD activity. Thus, our findings strongly suggest that upon PDT with HPD the mild hyperthermia (~ 44 °C) produced by photoirradiation might enhance the PDT-induced oxidative stress and, as a result, its tumoricidal effect *via* a rise in the activity of XOD. Besides, the obtained results suggest that severe hyperthermia (> 45 °C) could induce, contrary to mild hyperthermia, a reduction in the efficiency of HPD-PDT; we found that in EAC cells the raising temperature of an environment from 30 to 44 °C induced more than 2-fold increase in the activity of XOD, whereas further heating from 44 to 60 °C led to inactivation of this enzyme.

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

PDT* involves administration of a tumor-localizing agent (photosensitizer) 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 of tumor tissues. Preclinical and clinical studies conducted worldwide over a 30-year period have established PDT as a useful approach for treatment of some tumors [1]. HPD, commercially known as Photofrin, is still the most widely used PS in PDT of malignancies. The effectiveness of HPD-PDT depends largely on the presence of oxygen [2], and it was reported [3] that $^1O_2$ (a highly active oxidant) is the main damaging agent in PDT. At the same time, some *in vitro* [4–6] and *in vivo* studies [7–9] strongly suggest that other ROS, such as H_2O_2, $O_2^{\bullet}$ and $OH^{\bullet}$ radicals, could also participate in the HPD-PDT-induced tumor eradication. The rapid tumoricidal response observed following HPD-PDT results from oxidative injury to both the treated tumor cells and the exposed vasculature [10]. It was discovered that local immune reactions, e.g. macrophages, could be also responsible for the tumor necrotizing effect induced by HPD-PDT [11].

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Abbreviations: BSA – bovine serum albumin; EAC – Ehrlich ascites carcinoma; HPD – hematoporphyrin derivative; H_2O_2 – hydrogen peroxide; NBT – nitro blue tetrazolium; $^1O_2$ – singlet oxygen; $O_2^{\bullet}$ – superoxide radical; $OH^{\bullet}$ – hydroxyl radical; PS – photosensitizer; PBS – phosphate-buffered saline; PDT – photodynamic therapy; PrSH – protein-bound sulfhydryl groups; ROS – reactive oxygen species; SE – standard error; TB – trypan blue; XDH – xanthine dehydrogenase; XOD – xanthine oxidase; XOR – xanthine oxidoreductase.

Although to date PDT with HPD has already proved its effectiveness in the treatment of cancer, the molecular mechanisms by which this therapy destroys tumor cells as well as its optimal physical parameters are still incompletely understood. Clinical trials showed that during a standard regimen of PDT a considerable (5-13 °C) increase in the temperature of tumor tissues can take place [12]. Studies on various experimental tumors clearly demonstrated that the 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 tumor cells [13–15]. However, until now the mechanism of the synergism has been studied insufficiently, though these observations led to 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 [14]. It was suggested that in PDT with HPD the hyperthermia produced by a laser irradiation could improve the tumor response to the therapy *via*: inhibiting the repair of photodynamically induced injuries [16], an increase in the reactivity of the formed $^1O_2$ [17], an inactivation of antioxidant defenses of tumor cells [18] as well as by improvement of the oxygenation status of tumors [19]. At the same time, in the previous study we found that potentiating effect of heat shock on the antitumor efficiency of HPD-PDT could be largely explained by the stimulation of H_2O_2, $O_2^{-\bullet}$ and $OH^\bullet$ formation [20]. Our investigations showed also, that the stimulatory effect of heating on 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 even other pathways which could mediate the enhancing effect of heat stress on generation of H_2O_2 and oxygen radicals in tumor cells subjected to HPD-PDT.

In mammals, one of the major sources of ROS is XOR that catalyzes the last two reactions of purine catabolism by oxidizing hypoxanthine to xanthine and further to uric acid. This enzyme exists in two interconvertible forms, XDH and 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 [21]. As a result of its ability to generate ROS, XOD has received considerable attention as a pathological cause of ischemia-reperfusion injury [21], tissue damage associated with the inflammatory response [22], and cutaneous photosensitivity to hematoporphyrins [7]. Elevation of XOD activity during carcinogenesis including promotion phase was also reported [23]. Recent *in vitro* [4] and *in vivo* studies [9] showed that photosensitization of tumor 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 the antitumor effect of this photochemotherapy. In fact, in an earlier study we revealed that *in vitro* photodynamic treatment of EAC cells by HPD resulted in a considerable increase in the intracellular content of $O_2^{-\bullet}$ that was associated with a powerful (almost 3-fold) elevation in the activity of XOD; however, the phototoxic action of the PS towards the cells as well as the formation of $O_2^{-\bullet}$ were substantially suppressed upon addition of allopurinol [4], an effective and specific inhibitor of XOD. Further, KORBELIK and coworkers, using mice with subcutaneously transplanted FsaR fibrosarcoma, have found that photosensitization of the tumor 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 tumor response to this phototherapy [9]. It was reported that XDH can be reversibly converted to XOD by heating [24]. 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 tumor cells. However, this hypothesis requires an experimental checking.

Thus, the main aim of this work was to clarify the mechanism of the stimulatory effect of heat stress on generation of $O_2^{-\bullet}$ (a precursor of H_2O_2 and very cytotoxic $OH^\bullet$) in tumor cells subjected to PDT with HPD. This information is needed for more precise understanding the mechanism of the potentiating effect of heating, associated with the absorption of optical radiation, on the antitumor efficiency of PDT.

Materials and methods

Chemicals. Allopurinol, BSA (fatty acids free), isoxanthopterin and all other chemicals (of analytical grade or better) were purchased from Sigma, St. Louis, MO, USA. HPD was prepared from hematoporphyrin dihydrochloride as described in [25].

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. 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 (~ 2.5 x 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 (the range between 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. 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 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) exactly as described in our previous work [4] and kept in an ice bath until use. For the simultaneous thermal and PDT treatment, the cells loaded with HPD were resuspended in pre-heated PBS at a concentration of 5 x 10^6 cells/ml. An 8 ml sample of the cell suspension was quickly transferred into a 2 x 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 errors limits of ± 0.5 °C. The time interval between 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 TB exclusion assay as described in [20].

Assay for $O_2^{-\bullet}$ generation. The formation of $O_2^{-\bullet}$ in control and HPD-PDT-treated EAC cells was estimated by the NBT method [26] essentially as described previously [4]; the reduction of NBT by $O_2^{-\bullet}$ leads to the formation of a blue-colored formazan that was quantified spectrophotometrically. Briefly, HPD-loaded EAC cells (5 x 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 x 10^6 cells.

Quantification of XOD and total XOR activities in EAC cells. XOD and total XOR activities were measured in cell lysates at 37 °C by a very sensitive fluorometric assay [27] with minor modifications. At different times after photodynamic treatment, EAC cells (~ 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. XOD activity 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 the total (XOD + XDH) XOR activity. The reaction was then stopped by addition of allopurinol to a final concentration of 10 μM. The fluorescence at 390 nm was measured before and after addition of 15 μl of 10 μM isoxanthopterin to the assay mixture as an internal standard. XOD and XOR activities 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.

Determination of the intracellular content of PrSH. This was carried out by the Ellman method with 5,5'-dithiobis-2-nitrobenzoic acid exactly as described in [28].

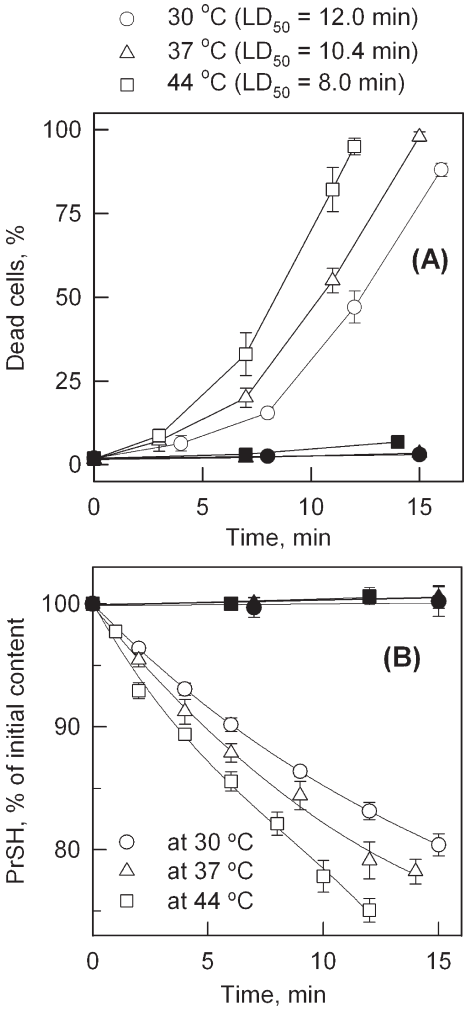

Figure 1. Kinetics of inactivation of HPD-loaded EAC cells (5 x 10^6 cells/ml in PBS) (A) as well as the content of PrSH in the cells (B) during their photoirradiation (opened symbols) or incubation in the dark (filled symbols) at different temperatures. The initial content of PrSH in the non-irradiated 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.

Statistics. Results were analyzed statistically by the Student's t-test. Values of $P < 0.05$ were considered statistically significant. Data in the text and figures are presented as mean ± SE of at least three separate experiments.

Results and discussion

In our 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. Figure 1A shows that

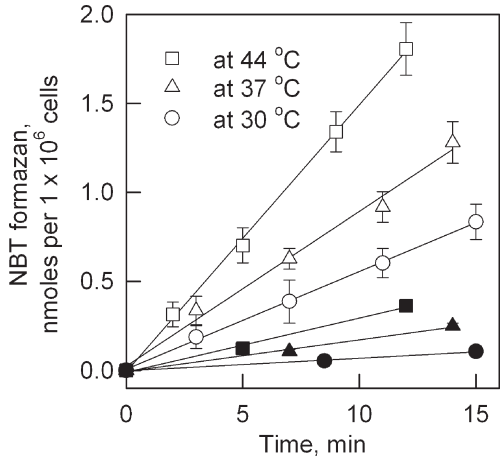

Figure 2. The formation of $O_2^{\cdot-}$ in HPD-loaded EAC cells (5 x 10^6 cells/ml in PBS) during their photoirradiation (opened symbols) or incubation in the dark (filled symbols) at different temperatures. Bars are SE.

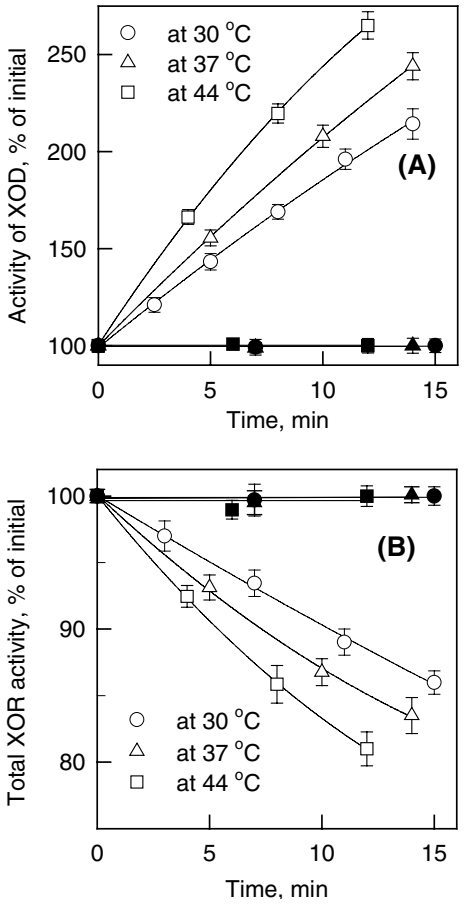

Figure 3. XOD (A) **and total XOR** (B) **activities in HPD-loaded EAC cells (5 × 10^6 cells/ml in PBS) during their photoirradiation (opened symbols) or incubation in the dark (filled symbols) at various temperatures. The initial XOD and total XOR activities in the non-irradiated EAC cells were measured as 11.7 ± 0.7 and 36.4 ± 1.8 pmoles isoxanthopterin formed/min per mg cell protein, respectively. Bars are SE.**

under photoexcitation of HPD, the kinetic of EAC cells inactivation was of the S type; after a lag period, 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 was utilized as a criterion to evaluate the efficiency of HPD-PDT-induced cytotoxicity. 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. 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 (~ 1.7-fold) growth in the rate of PrSH photooxidation was monitored (Fig. 1B). However, heat shock itself did not induce similar changes in the composition of cell proteins. Thus, our data support the view [13–15, 29] that in laser photochemotherapy the mild hyperthermia (around 44 °C) produced by irradiation can enhance synergistically the HPD-photoinduced tumor eradication.

Heat stress during PDT could enhance the HPD-induced photooxidative damage of cellular constituents and, as a consequence, killing of tumor cells not only *via* an increase in the reactivity of $^1O_2$ [17], but also through the stimulation of other ROS formation. In fact (Fig. 2), we found that upon HPD-PDT a rise in the temperature from 30 to 44 °C strongly (by ~ 2.5-fold) enhanced the generation of $O_2^{\cdot-}$ in EAC cells that correlated well with an increase in the rate of their photosensitized inactivation.

In order to clarify the mechanism of the catalyzing effect of heating on the formation of $O_2^{\cdot-}$, we examined the influence of temperature on the activity of XOD in HPD-loaded EAC cells upon their photoirradiation or incubation in the dark; in the previous *in vitro* study we established that in EAC cells subjected to the phototoxic action of HPD a part of the formed $O_2^{\cdot-}$ is generated by the ROS producing enzyme and that this oxygen radical is involved in killing of these cells [4]. Experiments showed that heating contributed to the PDT-induced rise in the activity of XOD; namely, the 12 min treatment of EAC cells by HPD-PDT at a 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. 3A). 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 hyperthermic (44 °C) temperature had no effect on the activity of XOD (Fig. 3A). The conversion of XDH to XOD may occur reversibly through oxidation of sulfhydryl groups or irreversibly by limited proteolysis [21]. In the prior work, we revealed that in tumor cells treated with HPD-PDT an increase in the activity of XOD is induced by the conversion of XDH to its oxidase form, pre-

dominantly, *via* the oxidation of sulfhydryl groups in XDH [4]. At the same time, experiments on EAC cells showed that heating caused a strong increase the rate of HPD-photosensitized oxidation of cysteine thiols in cell proteins (Fig. 1B). 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-catalyzed 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 of the cells (Fig. 3B). 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 tumor cells XDH is persistent to HPD-PDT-induced inactivation.

Our investigations showed that heat stress itself can enhance the generation of ROS in tumor cells. In fact, 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 $O_2^{\cdot}$ production by the cells (Fig. 2). The finding is in good agreement with the observation of other researchers [30], who discovered that heating (from 37 to 43-45 °C) of non-transformed as well as tumor cells strongly enhances the generation of oxygen radicals in the cells. Until now, the precise location and mechanisms of increased formation oxygen-derived free radicals upon heat stress are studied insufficiently. Nevertheless, there are some indications that the mitochondrial electron transport chain could be responsible for an increased production of $O_2^{\cdot}$ and H_2O_2 in cells subjected to a thermal stress [31]. As in tumor cells XOD is found [4] to be involved in the generation of ROS, we assumed that heating could elevate the activity of the enzyme in the cells and, thereby, formation of $O_2^{\cdot}$. In this connection, we estimated 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 2-fold increase in the activity of XOD in the cells; however, further heating from 44 to 60 °C resulted in inactivation of this enzyme (Fig. 4). Thus, our studies strongly suggest that XOD may be responsible for an increased formation of $O_2^{\cdot}$ and H_2O_2 in tumor 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 tumors.

In conclusion, the major finding of our studies is that upon HPD-PDT the hyperthermia produced by photoirradiation might enhance the PDT-induced oxidative stress and, as a result, its tumoricidal effect through a rise in the activity of XOD. Upon PDT the temperature of tumor lesions is commonly not controlled. To our mind, a tight control of tumor surface temperature during HPD-PDT, especially when it is combined with simultaneous local hyperthermia, is needed to obtain the maximal value of tumor necrosis. Indeed, our findings as well as some data from the literature suggest that

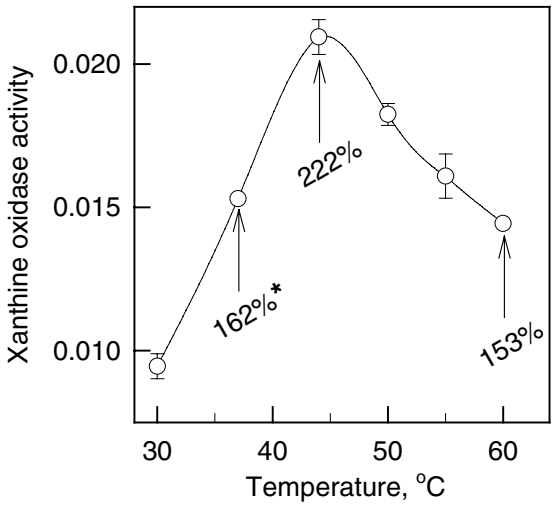

Figure 4. Influence of temperature on the activity of XOD in intact EAC cells. The enzyme activity is expressed as nmoles isoxanthopterin formed/min per mg cell protein. *, % from the value obtained at 30 °C. Bars are SE.

severe hyperthermia (at temperatures > 45 °C) could induce, contrary to mild hyperthermia (around 42–44°C), a reduction in the antitumor 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 tumor tissues [2]. However, it was reported that severe hyperthermia may cause a drastic drop in tumor oxygen tension due to an almost complete cessation of nutritive blood flow [19].

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