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The effect of photodynamic treatment on the activity of regulatory glycolytic enzymes in A431 cells

Virginija Sabaliauskienė*,

Aušra Sasnauskienė,

Jurgis Kadziauskas,

Vida Kirvelienė

Department of Biochemistry and Biophysics, Vilnius University, M. K. Čiurlionio 21, LT-03101 Vilnius, Lithuania

The effect of photodynamic treatment on the activity of regulatory glycolytic enzymes hexokinase (EC 2.7.1.1) and pyruvate kinase (EC 2.7.1.40) has been investigated. The experiments were carried out on human epidermoid carcinoma A431 cells photosensitised with either safranin O (Safr) or mTHPC. An increased concentration of ATP in the treated cells has been observed. The Safr-mediated treatment at CD80 and mTHPC-mediated treatment at CD50 induced an immediate sustained significant reduction of hexokinase and pyruvate kinase activities. No changes were found in the activities of both enzymes when cells were treated with Safr at CD50. The activities of HK and PK were reduced by PDT under conditions involving damage to mitochondrial outer membrane.

Key words: photodynamic treatment, photosensitisation, ATP, A431 cells, hexokinase, piruvate kinase

Abbreviations: CD, cytotoxic dose; HK, hexokinase; mTHPC, *meso*-tetra(3-hydroxyphenyl)-chlorine; PDT, photodynamic treatment; PEP, phosphoenolpyruvic acid; PFK, phosphofructokinase; PK, pyruvate kinase; ROS, reactive oxygen species, Safr, safranin O (3,7-diamine-2,8-dimethyl-5-phenylphenazine chloride), TEA, triethanolamine.

INTRODUCTION

In our study, attention was focused on the activity of regulatory glycolytic enzymes in photodynamically treated cells. PDT is a treatment mode for some malignant and non-malignant diseases caused by cellular overgrowth. It is based on the excitation of a cell-localized photosensitiser with visible light and the following generation of ROS. The produced ROS induce an oxidative damage to the cellular components that accumulate the photosensitiser and thus might lead to cell death by apoptosis, necrosis, and other death paths (for a review, see Ref. 1). Mitochondria were shown to be important targets for PDT [2]. The damage to mitochondria can impair ATP production. However, in our previous investigation, we observed a slight increase of the cellular ATP level following mitochondria-involving PDT [3]. Since ATP can be produced by glycolysis, in this study we have investigated the activity of glycolytic enzymes in photodynamically treated cells.

The key enzymes in the regulation of glycolysis are hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK). These three enzymes have a potential rate-limiting activity and do not achieve equilibrium but are associated with a large negative free energy change and are therefore effectively irreversible. The same three enzymes have the largest flux control coefficient C which measures the impact of a change in the activity of the enzyme on the flux of reactants through the whole pathway [4]. The activity of PK is regulated allosterically, being inhibited by ATP and stimulated in a feed-forward mechanism by fructose-1,6–bisphosphate (the product of PFK-catalyzed reaction). It is noteworthy that both HK and PFK use ATP, while PK produces ATP.

The aim of the present study was to investigate the PDT effect mediated by mitochondrially localised Safr [5] and membranously (including mitochondrial membranes) localised mTHPC [6] on the activity of the glycolytic enzymes hexokinase and pyruvate kinase in A431 human squamous cell carcinoma cells.

MATERIALS AND METHODS

Materials

DMEM, FCS and streptomycin were obtained from Biochrom AG, Germany. mTHPC (a generous gift of R. Bonnett, Queen Mary, University of London, UK) was dissolved in ethanol as 1 mg/ml stock solution and stored at –20 °C in the dark. The stock solution of Safr (Sigma) was prepared in the same way. All experiments were performed using dilutions of the stock solutions with cell incubation media. Enzymes and substrates: glucose-6-phosphate dehydrogenase (Boehringer Mannheim), HK, PK, phosphoenolpyruvic acid potassium salt (Fluka); inhibitors of proteases: aprotinin, bacitracin, EDTA, PMSF (Roth). Other chemicals were from Sigma. Culture flasks and Petri dishes were from TPP, Switzerland.

Cell culture

A-431 human squamous cell carcinoma cells (DSMZ, Germany) were cultured in monolayer in 75 or 150 cm^2 flasks in DMEM

* Corresponding author. E-mail: virginija.sabaliauskiene@gf.vu.lt

supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 5 mM HEPES, at 37 °C in a humidified atmosphere with 5% CO_2. Cells were subcultured by dispersal with 0.025% trypsin in 0.02% EDTA and replated at a density of 1×10^5 cells/ml twice a week.

Photodynamic treatment of cells

Safr-PDT. Cells were seeded at a density of 1×10^5 cells/ml. After 48 h, the cells were washed twice with DPBS, and FCS-free DMEM supplemented with 0.77 μg/ml Safr was added. Following incubation for 1 h, the cells were washed twice with DPBS, and DMEM supplemented with 5% of FCS was added. The cells were exposed to the light of the LED array UNIMELA-2 (Vilnius University Laser Research Centre, Lithuania) at $\lambda = 509 \pm 5$ nm (29 W/m^2) for 3 and 10 min and incubated in the dark at 37 °C in a 5% CO_2 atmosphere.

mTHPC-PDT. Cells were seeded at a density of 1.2×10^5 cells/ml. After 30 h, the cells were washed twice with DPBS buffer, and FCS-free DMEM containing 0.1 μg/ml mTHPC was added for 18 hours. After incubation, the cells were washed twice with DPBS, and DMEM containing 5% of FCS was added. The cells were exposed to the light of the LED array UNIMELA-1 (Vilnius University Laser Research Centre, Lithuania) at $\lambda = 660 \pm 5$ nm (16 W/m^2) for 1, 1.5 and 3 min and incubated in the dark at 37 °C in a 5% CO_2 atmosphere.

Cell viability assay

After light exposure, the cells were incubated for 24 h until cell viability was estimated by staining with crystal violet. Briefly, the cells were fixed with 96% ethanol for 10 min, 0.05% crystal violet solution in 20% ethanol was added for 30 min, the cells were rinsed, the remaining cell-attached dye was dissolved in 0.1% acetic acid solution in 50% ethanol, and the optical density was recorded at 585 nm [7].

Lysis of cells

The extraction buffer contained 1.872 ml of PBS, 6 μl of 0.5 mM EDTA, 8 μl of 0.5 mM EGTA, 2 μl of bacitracin (20 μg/ml), 2 μl of aprotinin (0.4 mg/ml), 10 μl of 200 μM PMSF in 2-propanol, 100 μl of 20% triton X100. The final volume of the extraction buffer was 2 ml. The cells in 300 μl of the extraction buffer were incubated in an ice bath for 20 min and then centrifuged for 15 min at 4 °C (10 000 × g). The supernatant was used for the estimation of HK and PK activity.

Determination of hexokinase activity

The method of determination is based on HK reaction coupled to $NADP^+$ reduction in glucose-6-phosphate dehydrogenase-catalyzed reaction. The assay mixture contained 2.2 ml of 50 mM Tris-HCl (pH 8) with 13.3 mM $MgCl_2$ (Tris-$MgCl_2$), 0.5 ml of 0.67 M glucose in Tris-$MgCl_2$, 100 μl of 16.5 mM ATP in Tris-$MgCl_2$, 100 μl of 6.8 mM $NADP^+$ in Tris-$MgCl_2$, 5 μl of glucose-6-phosphate dehydrogenase (300 U/ml, diluted at 1 : 8 with Tris-$MgCl_2$). After measuring the blank-reaction rate (if such reaction did occur) for 2–3 min, 100 μl of cellular extract was added and the increase in absorbance was recorded for 10 min at 340 nm, 30 °C. The enzyme amount reducing 1 μmol of $NADP^+$ during 1 min under the conditions specified was taken for the activity unit. The activity of HK was calculated using the following formula:

$$\text{U/mg protein} = \frac{\Delta A_{340}/\text{min}}{6.22 \times \text{mg protein/ml react mix}}.$$

Determination of pyruvate kinase activity

The activity was estimated by measuring NADH oxidation in a coupled assay with lactate dehydrogenase. The assay mixture contained 2.5 ml of 8.3 mM TEA (pH 7.5), 100 μl of 2.25 M KCl, 100 μl of 0.24 M $MgSO_4 \times 7\ H_2O$, 100 μl of 24 mM ADP, 100 μl of 5.7 mM NADH, 10 μl of 45 mM PEP and 20 μl of LDH (85 U/mg). The enzyme amount oxidizing 1 μmol of NADH during 1 min under the conditions specified was taken for the activity unit. Calculations were performed in the same manner as in the case of HK.

ATP assay

The cellular ATP content was measured using the CytoPro Kit (Labsystems) based on the luciferin-luciferase reaction [8]. Luminescence measurements were made using a microplate reader Ascent FL (Labsystems). ATP was normalised to protein content in a sample.

Determination of protein concentration

Protein concentration was determined using bicinchoninic acid sodium salt (4,4'-dicarboxy-2,2'biquinoline disodium salt) (BCA) [9]. The optical density at 562 nm was recorded on a Perkin–Elmer spectrometer Lambda 20.

Data analysis

The data are presented as means ± standard deviation (SD) from three independent experiments.

RESULTS AND DISCUSSION

Changes in viability of photosensitizied cells. Photosensitisers that localise in different cellular compartments were employed. Safr is uptaken to the mitochondrial matrix with regard to the negative potential of the mitochondrial inner membrane [5], and hydrophobic mTHPC is localised at the membranes of the endoplasmic reticulum, Golgi apparatus, and mitochondria [6].

The viability of Safr-PDT cells after light exposure for 3 min decreased by 50% (CD50) and after 10 min approx. by 80% (CD80). In the case of mTHPC, light exposure for 1, 1.5 and 3 min led to a decrease of viability by 35%, 50% (CD50) and 80% (CD80), respectively (viability results not shown). Since the concentration of mTHPC was lower than that of Safr, it should be concluded that mTHPC was a considerably more effective photosensitiser than Safr.

Changes of ATP concentration in A431 cells following PDT. Both Safr- and mTHPC-mediated PDT induced a slight but significant increase of the intracellular ATP level in A431 cells, which was registered up to two hours after light exposure (Fig. 1). In some experiments the ATP concentration reached 150% of that in the intact control cells. The increase of ATP can be caused by two alternatives: either ATP production is upregulated or ATP consumption is decreased. The high ATP level observed in our experiments could not be explained by the inhibition

of ATP-consuming processes alone, since in the absence of substrates of energy metabolism in photodynamically treated MH cells, the ATP pool was depleted within 2 h following PDT [10]. Therefore, in this study, we investigated the possibility of the upregulation of ATP synthesis. Since both photosensitisers localise to mitochondria and could induce their damage, it was assumed that ATP synthesis could not be upregulated by activating oxidative phosphorylation. Thus, we tested the hypothesis that increased ATP could be produced by glycolysis which is a crucially important mechanism for the energetic metabolism of cancer cells [11]. Therefore, we examined the activity of the regulatory enzymes of glycolysis following PDT.

The effect of PDT on HK and PK activity. At CD50 of Safr-mediated PDT, the activity of HK and PK in cellular extracts was the same as that in extracts of intact control cells (Fig. 2). Safr-mediated PDT at CD80 caused a reduction of HK and PK activity by 44% and 53%, respectively. Changes in the activity of both enzymes were registered immediately after light exposure and for the next 2 h remained constant.

It should be noted that following Safr-mediated PDT at CD50, the cause of viability decrease was cell cycle arrest, and no hallmarks of apoptosis were observed (A. Sasnauskiene, unpublished results). It implies that cytochrome c and other apoptosis-inducing molecules did not escape mitochondria, and there was no considerable damage to the integrity of the mitochondrial outer membrane. It coincided with the high activity of the glycolytic enzymes. At CD80, Safr-mediated PDT was followed by a pronounced apoptosis, and it coincided with a significant drop-off in HK and PK activity.

A correlation between damage to the mitochondrial outer membrane and the reduction of the activity of glycolytic enzymes was observed in the cells following mTHPC-mediated PDT as well. Although no changes in the enzymatic activity was registered following Safr-mediated PDT at CD50, in the case of mTHPC-mediated PDT the activity of HK decreased by approx. 40% and that of PK by 20% (Fig. 3). Apoptosis is the prevailing cell death mode following mTHPC-mediated PDT under the applied conditions in the whole CD range [3] due to mTHPC accumulation in mitochondrial membranes. Since HK can exist in a mitochondrial membrane-bound form [12, 13], it is possible that the enzyme might be damaged by ROS produced during PDT under conditions when the damage to mitochondrial outer membrane occurs.

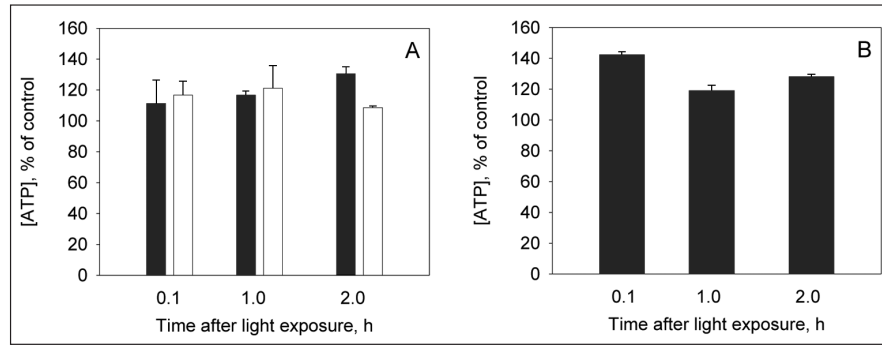

Fig. 1. ATP concentration in A431 cells after photodynamic treatment. A – Safr-PDT: cells were incubated for 1 h with 0.77 μg/ml Safr and exposed to light of 509 nm LED array for 3 min (black bars, CD50) or 10 min (white bars, CD80); B – mTHPC-PDT: cells were incubated for 18 h with 0.1 μg/ml mTHPC and exposed to light of 660 nm LED array for 1.5 min (CD50)

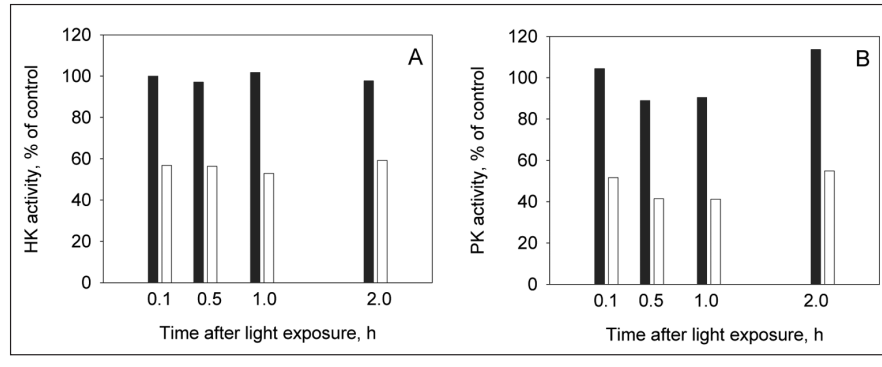

Fig. 2. HK and PK activities in A431 cells following Safr-mediated PDT. A – HK; B – PK. Cells were incubated for 1 h with 0.77 μg/ml Safr and exposed to light of 509 nm LED array for 3 min (black bars, CD50) or 10 min (white bars, CD80)

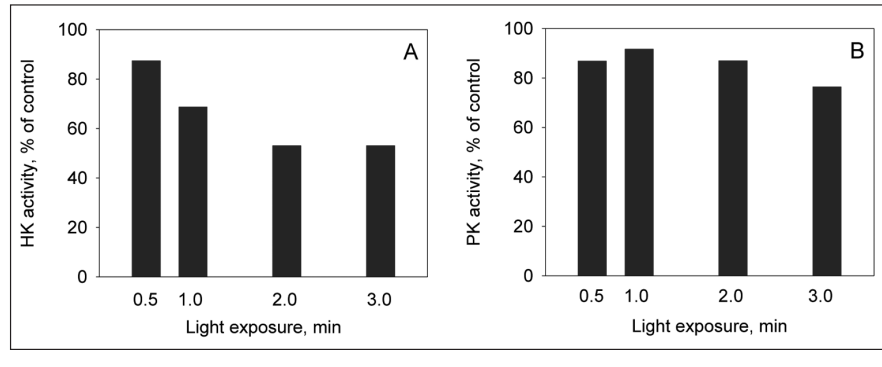

Fig. 3. The influence of light exposure on HK and PK activity in A431 cells following mTHPC-mediated PDT. A – HK; B – PK. Cells were incubated for 18 h with 0.1 μg/ml mTHPC and exposed to light of 660 nm LED array. Enzyme activity was determined at 0.5 h post-exposure

The inhibition of HK might result in a downregulation of PK activity. On the other hand, it should not be excluded that intensive photodynamic disturbances might cause a reduction of the cytosolic enzyme PK activity *per se*.

It should be admitted that the predicted increase of glycolytic enzyme activity, which could produce a rise of intracellular ATP levels, did not occur. The decrease of ATP consumption by injured cells following PDT might be the presumptive reason for ATP increase. However, the residual activity of enzymes in association with the decreased demand for ATP in the injured cells might contribute to the increase of ATP concentration.

CONCLUSIONS

The increased concentration of ATP in the A-431 human squamous cell carcinoma cells has been observed following the photodynamic treatment mediated by two photosensitisers, Safr and mTHPC. The activity of HK and PK was reduced by PDT involving a damage to mitochondrial outer membrane. A decrease of HK and PK activity took place immediately after light exposure or even during the exposure, and was sustained for 2 h post-exposure.

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Virginija Sabaliauskienė, Aušra Sasnauskienė, Jurgis Kadziauskas, Vida Kirvelienė

FOTODINAMINIS POVEIKIS GLIKOLIZĖS REGULIACINIŲ FERMENTŲ AKTYVUMUI A431 LĄSTELĖSE

Santrauka

Po fotodinaminio poveikio, kuriam naudotas fotosensibilizatorius safraninas O arba mTHPC, žmogaus epidermoidinės karcinomos A431 ląstelėse nustatyta padidėjusi ATP koncentracija. Siekiant nustatyti glikolizės ir padidėjusios ATP koncentracijos ryšį, buvo tirtas glikolizės reguliacinių fermentų – heksokinazės (EC 2.7.1.1) ir piruvato kinazės (EC 2.7.1.40) – aktyvumas po fotodinaminio poveikio. Nustatytas reikšmingas tirtų fermentų aktyvumo sumažėjimas iš karto po fotodinaminio poveikio, sukeliančio 80% ląstelių žūtį safranino O atveju bei 50% ląstelių žūtį mTHPC atveju. Safranino O fotodinaminiam poveikiui 50% sumažinus gyvybingumą, tirtų fermentų aktyvumas išliko nepakitęs. HK ir PK aktyvumo sumažėjimas nustatytas fotodinaminio poveikio sąlygomis, sukeliančiomis išorinės mitochondrijų membranos pažaidas.