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Differential effects of glucose deprivation on the cellular sensitivity towards photodynamic treatment-based production of reactive oxygen species and apoptosis-induction

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Abstract Photodynamic treatment (PDT) employs a photosensitizer and the light-induced formation of reactive oxygen species – antagonized by cellular antioxidant systems – for the removal of harmful cells. This study addresses the effect of altered carbohydrate metabolism on the cellular antioxidant glutathione system, and the subsequent responses to PDT. It is shown that glucose-deprivation of 18 h prior to PDT causes a reduced level of intracellular glutathione and an increased cytotoxicity of PDT. These effects can be mimicked by inhibitors of glutathione synthesis (buthionine-sulfoximine) or its regeneration (1,3-bis-(2-chlorethyl)-1-nitrosourea). Inhibited glutathione metabolism shifts the apoptotic window to lower fluences, while glucose deprivation abolishes apoptosis as a result of ATP deficiency. Our results prove evidence for manipulation of the outcome of PDT through internal metabolic pathways.

Keywords: Photodynamic treatment; Reactive oxygen species; Glutathione; Pentose phosphate pathway; Glycolysis; Apoptosis

1. Introduction

Reactive oxygen species (ROS) – although being an inevitable consequence of aerobic metabolism – pose a serious threat to the cellular integrity since they can oxidize almost every compound of biological origin and hence can cause severe cellular damage when produced over a certain level of quantity [1–3]. Photodynamic therapy (PDT) uses massive generation of ROS in target cells by application of photosensitizing molecules such as protoporphyrin or phthalocyanine derivatives and activation of these compounds by irradiation with light of the appropriate energy for the removal of harmful and unwanted cells [4]. By this, both modes of cell death, apoptosis and necrosis are induced in the target tissue (for review, see [5,6]).

To a certain extent, photodynamic therapy is antagonized by cellular antioxidant defense mechanisms. The antioxidative capacity of reduced glutathione (GSH) is employed to scavenge reactive oxygen intermediates yielding oxidized glutathione (GSSG), which can be regenerated to GSH by glutathione reductase under the expense of nicotine adenine dinucleotide phosphate reduced (NADPH) [7–9]. Among other carbohydrates, glucose and its metabolization in the first reactions of the pentose phosphate pathway (PPP) represent the most important source of NADPH [10,11]. From this knowledge, one might hypothesize that the availability of glucose and its metabolization in the PPP affects the cell's capability to regenerate GSH to the usual millimolar concentration range [10,12] and subsequently the cellular sensitivity towards PDT.

Besides the consequences of altered carbohydrate metabolism for the supply of NADPH, glucose deprivation (among other effects, see Section 4) also impairs cellular energetics by inhibition of glycolytic ATP production. This is of special importance for the mode of cell death induced by PDT: glycolytic ATP production may partly compensate for mitochondrial impairment during apoptosis and supply the ATP-requiring steps in active cell death [13–15]. We could prove this hypothesis in a set of experiments published recently, where glucose deprivation of photosensitized cells in vitro causes a rapid drop of ATP and subsequently an inhibition of apoptosis and a shift to necrotic cell death under conditions which normally would cause apoptosis [16]. An additional effect of impaired glycolytic ATP production may consist in reduced de novo synthesis of GSH, which requires two moles ATP per mole GSH [7,9].

By taking up these results, we expand the analysis of the effects of impaired carbohydrate metabolism to the possible influence on the antioxidative mechanisms during PDT. Employing a well established model system consisting of the

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Abbreviations: $AlPcS_4$, aluminum (III) phthalocyanine tetrasulfonate; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; BSO, DL-buthionine-sulfoximine; DCF, 2′-7′-dichlorofluorescein; DCFH-DA, 2′, 7′-dichlorodihydrofluorescein diacetate; FCS, fetal calf serum; (GF-)DMEM, (glucose-free) Dulbecco's modified Eagle's medium; GSH, reduced glutathione; GSSG, oxidized glutathione; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NADP(H), nicotine adenine dinucleotide phosphate (reduced); PBS, phosphate-buffered saline; PDT, photodynamic therapy; ROS, reactive oxygen species; PPP, pentose phosphate pathway

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hydrophilic sensitizer aluminum (III) phthalocyanine tetrasulfonate ($AlPcS_4$) and the human epidermoid carcinoma cell line A431, the present study addresses the question whether an altered carbohydrate metabolism would affect the cellular ROS antioxidative mechanisms and thus the susceptibility towards PDT. The increase in sensitivity to ROS caused by conditions of glucose deprivation could be causally attributed to impaired GSH regeneration or synthesis. However, as shown in this study, artificial inhibition of GSH metabolism did not influence the cell's ability to undergo apoptosis (in contrast to glucose-free conditions).

Aware of the fact that PDT is applied clinically in several countries for treatment of malignant and non-malignant diseases [4], this study proves the importance of the cellular (carbohydrate) metabolism for influencing the cellular susceptibility towards PDT and, therefore, the efficiency of the treatment regime.

2. Materials and methods

2.1. Cell culture and photodynamic treatment

A431 human epidermoid carcinoma cells (ATCC-Nr. CRL-1555) were cultured in Dulbecco's modified Eagle's Medium (DMEM; Sigma–Aldrich, Vienna, Austria) containing 4.5 g l^{-1} glucose supplemented with 10 mM HEPES, 4 mM L-glutamine, 1 mM Na-pyruvate, 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 5% fetal calf serum (FCS) (all from PAA-laboratories, Linz, Austria), in a humidified atmosphere at 37 °C and 7.5% CO_2. For measurement of caspase-3 like activity, nuclear fragmentation and ROS production, 3×10^5 cells were seeded into 30 mm petri dishes (Greiner Bio-One, Kremsmuenster, Austria); for measurement of cytotoxicity, 7500 and 12 500 cells were seeded into 96-well microplates (black walls, clear bottom; Greiner Bio-One) for samples cultured with or without glucose, respectively. Eighteen hours prior to PDT (i.e., 24 h after seeding the cells, in the log phase), the medium was replaced with 0% FCS DMEM (standard conditions, further referred to as 'DMEM') or – after a washing step with phosphate buffered saline (PBS) – with glucose-free Dulbecco's modified Eagle's Medium base (0% FCS, further referred to as 'GF-DMEM'; Sigma–Aldrich) supplemented as above containing 10 μM $AlPcS_4$ (product no. AlPcS-834; Frontier Scientific/Porphyrin Products, Logan, USA). When indicated, 3 mM DL-buthionine-sulfoximine (BSO; Fluka, Buchs, Switzerland) was added simultaneously with the sensitizer (18 h) and 500 μM 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, Sigma–Aldrich) was added 1 h prior to irradiation.

Immediately before irradiation, the culture supernatant was replaced by fresh 0% FCS medium (DMEM or GF-DMEM). Irradiation was performed using a red-light illumination diode array ($\lambda_{max} = 660 \pm 20$ nm, super bright diodes, product no. L53SRC-F from Kingbright Electronic, Issum, Germany) with a light power of 10 mW cm^{-2}. Samples were protected from ambient light; for all experiments cells from passage number 5–15 were used.

2.2. Cytotoxicity

Metabolic/mitochondrial activity was assessed by the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to the insoluble blue formazan catalyzed by mitochondrial and other cellular dehydrogenases [17]. For this purpose, cells were incubated for 23 hours after irradiation and the medium was replaced by 100 μl of DMEM for all samples to restore the carbohydrate metabolism of the cells kept in glucose-free medium, which has been proven in preliminary experiments (data not shown) to allow sufficient conversion of MTT. After another hour, 10 μl of a solution containing MTT (5 mg ml^{-1} in PBS) was added to the microplate wells. Reduction of MTT was allowed to proceed for 45 min; afterwards the medium was removed, the formazan dye was solubilized by addition of 100 μl 2-propanol and the absorbance was measured at 565 nm using a Spectrafluor microplate reader (Tecan, Salzburg, Austria).

2.3. Measurement of intracellular glutathione

Intracellular GSH was extracted and measured based on a protocol published by Griffith [18]. In short, cells were cultured in 60 mm petri dishes (7.5×10^5 cells/3 ml medium) under conditions described above (DMEM, BSO, BCNU, GF-DMEM; all without sensitizer). At that time where the samples are – otherwise – PDT-treated, the supernatant was removed, the cells were washed twice with PBS and harvested by trypsinization. Dilution series of the cell samples as well as of GSH (Sigma–Aldrich) were made in PBS and extracted with 1 volume 5-sulfosalicylic acid (5%, w/v); cell debris was removed by centrifugation and the GSH released from the cells was measured by addition of 5-5′-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent, absorbance at 405 nm; Spectrafluor reader, Tecan). From estimation of the cell diameter and cell number by means of an electronic cell counter (CASY-1, Schaerfe-Systems, Reutlingen, Germany; usually in the range of 19 μm), the intracellular concentration of GSH, $[GSH]_{ic}$, was calculated.

2.4. Measurement of intracellular ROS

The measurement of intracellular ROS is based on the oxidation of DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate), which is quantified by flow cytometry [1]. In brief, 30 min prior to irradiation, cells (treated with BSO/BCNU or GF-DMEM as described above) were incubated with 50 μM DCFH-DA. Twenty minutes after irradiation, cells including the supernatant were harvested using Accutase (PAA-laboratories); all subsequent steps were performed on ice. The cells were spun down at 840 ×*g* and washed once with PBS; after another centrifugation step, the pellet was resuspended in 500 μl PBS and the resulting red fluorescing DCF (2′–7′-dichlorofluorescein) was analyzed by flow cytometry (FACS Calibur, Beckton–Dickinson, Franklin Lakes, NJ, USA). For each sample, 10 000 cells were analyzed; the fluorescence signal (FL2 channel, $\lambda_{EX} = 488$ nm, $\lambda_{EM} = 580 \pm 40$ nm) was evaluated as discussed in Section 3.

2.5. Apoptosis detection (I): caspase-3 assay

As a central indicator of apoptotic cell death, 5 h post-treatment the activity of caspase-3 like proteases was analyzed by means of a fluorigenic peptide. For experimental details, see [16]. The results are related to the protein content to correct for variations in cell number assayed; finally, the data are shown as percentage of an UV-treated sample (200 mJ cm^{-2}, $\lambda_{max} = 254$ nm, Stratagene's Stratalinker, Amsterdam, Netherlands), which is referred to induce a homogenous apoptotic population of cells [19].

2.6. Apoptosis detection (II): nuclear fragmentation

Nuclear fragmentation during apoptosis is measured by analysis of the DNA content. This method is based on the flow cytometric analysis of the DNA histogram of ethanol-fixed, ribonuclease A-treated and propidium iodide (PI) stained cells, whereby cells characterized by a smaller fluorescence than the G_1 peak were considered as apoptotic cells ('Sub-G_1 peak'). For the detailed protocol, see [16]; for each sample, 10 000 cells were analyzed.

2.7. Statistical evaluation

Mean values of three independent experiments ± S.E.M. or Gaussian Errors are shown throughout. Statistical significance was evaluated using Student's *t* test.

3. Results

3.1. Cellular sensitivity towards PDT and its relationship to glucose/glutathione metabolism

In order to determine the efficiency of the PDT protocol applied, we employed the MTT assay which gives information on metabolic/mitochondrial activity and hereby allows an estimation of the overall cytotoxicity [20].

Fig. 1 shows the percentage of the MTT activity of standard PDT samples (DMEM), cells grown under glucose-free conditions (GF-DMEM) and those treated with inhibitors of the glutathione metabolism (BSO or BCNU) as related to the

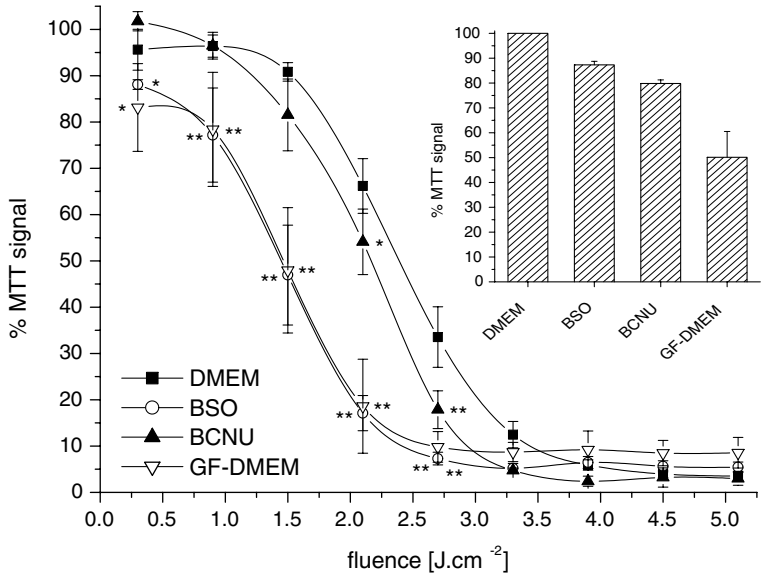

Fig. 1. Survival of photosensitized A431 cells under conditions of impaired carbohydrate/glutathione metabolism. PDT-induced cytotoxicity was assessed 24 h p.i. by means of MTT assay (Section 2.2). Results are expressed as relative MTT activity of untreated control samples; these controls (treated with BSO/BCNU/GF-DMEM but without PDT) are shown in the insertion. When indicated ('BSO'/'BCNU'), 3 mM buthionine sulfoximine was added 18 h and 500 μM 1,3-bis-(2-chloroethyl)-1-nitrosourea was added 1 h before illumination, respectively; GF-DMEM indicates glucose free medium. Mean values from three independent experiments (error bars as calculated by the Gaussian law of error propagation). Highly significant ($P < 0.01$)/significant ($P < 0.05$) differences compared to the standard PDT samples (DMEM) are indicated by ∗∗ and ∗, respectively.

respective controls (non-irradiated, without $AlPcS_4$, but similarly treated with GF-DMEM/BSO/BCNU). These controls showed the following MTT activities: DMEM 100%, BSO 87.3% ± 1.4, BCNU 79.8% ± 1.4 and GF-DMEM 50.1% ± 10.4 (see insertion in Fig. 1). Preceding control experiments [16] showed that this reduction is not due to cell killing by the media composition itself, but caused by reduced proliferation (data not shown). Furthermore, at a concentration of 10 μM, dark controls (data not shown) demonstrated $AlPcS_4$ not to be toxic.

All curves are characterized by a steep decrease in MTT activity within about a 2 J cm^{-2} change in light fluence ranging from 90% to 100% MTT activity to about 10% at higher light doses. Although similarly shaped, the curves for the glucose-free sample as well as those of cells with impaired GSH metabolism are clearly shifted to lower light doses as compared to the standard PDT treated cells (DMEM): a shift of about 1 J cm^{-2} is found for glucose free samples and those treated with BSO; a smaller change (approximately 0.5 J cm^{-2}) is found for BCNU treated samples.

3.2. Intracellular GSH

The intracellular concentration of GSH at the time of PDT is shown in Fig. 2. Treatment with BSO (3 mM, 18 h) reduced $[GSH]_{ic}$ from 6 mM (DMEM) to 1 mM; in contrast, the GSH level of cells treated with BCNU was not significantly reduced. The concentration of GSH in samples deprived of glucose for 18 h was nearly reduced to half (3 mM).

3.3. Generation of ROS by PDT

Determination of intracellular ROS was employed in order to clarify whether the altered cellular sensitivity caused by glucose depletion or inhibition of GSH metabolism by BSO or BCNU is caused by an alteration of ROS generation following

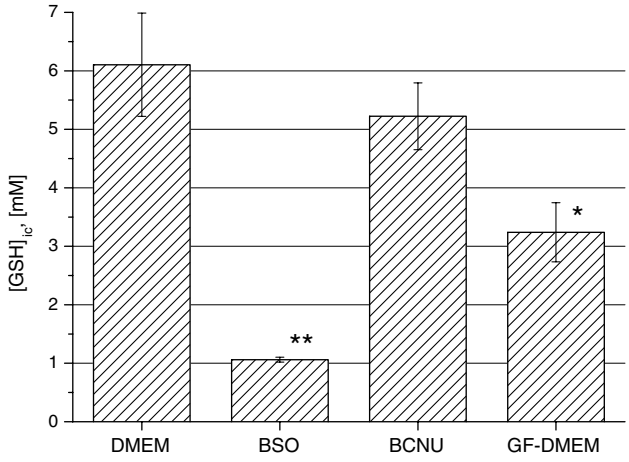

Fig. 2. Intracellular GSH under different metabolic conditions. Intracellular GSH concentration ($[GSH]_{ic}$, mM) was measured for samples with different media and additives (3 mM BSO (buthionine sulfoximine) or glucose-free medium (GF-DMEM) for 18 h and 500 μM BCNU (1,3-bis-(2-chloroethyl)-1-nitrosourea) for 1 h). Mean values of three independent measurements ± S.E.M. Highly significant ($P < 0.01$)/significant ($P < 0.05$) differences compared to the control sample (DMEM) are indicated by ∗∗ and ∗, respectively.

PDT. The level of intracellular ROS is changed even under control conditions (without PDT, see Fig. 3); for samples treated with GF-DMEM/BSO/BCNU but without PDT, a DCF signal for glucose-free samples and those treated with BSO or BCNU of about 58, 39, and 22 rfu (relative fluorescence units), respectively, can be found compared to control (DMEM, ~13 rfu).

For PDT, the dynamics of ROS generation in standard PDT samples (DMEM) is characterized by an increase of intracellular ROS with a small slope at low light fluences (0.25–1 J cm^{-2})

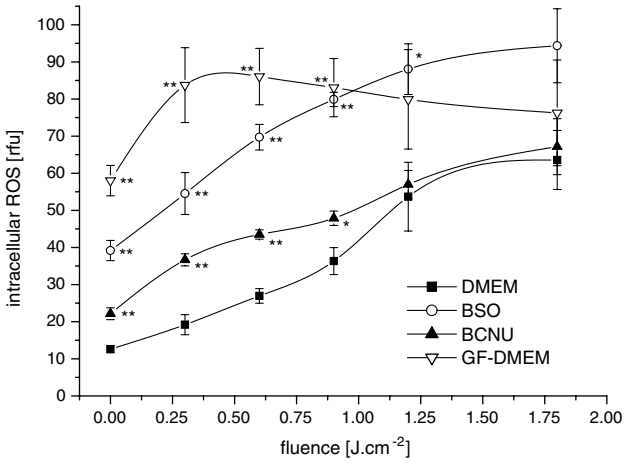

Fig. 3. Generation of intracellular ROS by photodynamic treatment. Intracellular ROS were determined by flow cytometric analysis of DCFH-DA oxidation (Section 2.4). When indicated ('BSO'/'BCNU'), 3 mM buthionine sulfoximine was added 18 h and 500 μM 1,3-bis-(2-chloroethyl)-1-nitrosourea was added 1 h before illumination, respectively; GF-DMEM indicates glucose free medium. The first data points ('0 J cm^{-2}') refer to untreated controls (treated with BSO/BCNU/GF-DMEM but without PDT). Data, given as rfu (relative fluorescence units), are representative of three independent experiments ± S.E.M. Highly significant ($P < 0.01$)/significant ($P < 0.05$) differences compared to the standard PDT samples (DMEM) are indicated by ** and *, respectively.

and an increasing ROS level at higher fluences (Fig. 3). Deprivation of glucose causes a high level of DCFH-DA oxidation already at low doses, i.e., an increased level of intracellular ROS is observed within the whole range of PDT doses. Inhibition of GSH synthesis or reduction by BSO and BCNU, respectively, results in a gradual elevation of ROS generated by PDT, although the effect is more pronounced for BSO-treated samples.

At high light doses (>1.75 J cm^{-2}), an additional peak appears in the DCF histogram which shows considerable lower fluorescence than the untreated control samples (about 1 order of magnitude lower; data not shown). These cells were not included in the analysis, since this peak contains most probably necrotic cells characterized by reduced membrane integrity which caused the fluorescent dye to leak into the medium.

3.4. Induction of apoptosis by PDT and its relationship to glucose/glutathione metabolism

Fig. 4A shows the activity of caspase-3 like enzymes 5 h p.i. depending on the light fluence. For standard PDT treated cells, caspase-3 activation peaked at a fluence of 2 J cm^{-2} (non-irradiated controls without sensitizer are indicated by a light dose of '0' in the diagram). The maxima of caspase-3 like activity of samples treated with BSO or BCNU are clearly shifted to lower irradiation doses of about 1.5 J cm^{-2}. For glucose-free cells, no significant activation of capase-3 like enzymes can be found.

Results from determination of the nuclear fragmentation confirm these findings: as shown in Fig. 4B, maxima of nuclear fragmentation are present at 2 and 1.5 J cm^{-2} for standard PDT samples and cells treated with BSO/BCNU, respectively; again, impairment of the GSH metabolism results in a shift of the 'apoptotic window' to lower irradiation doses. In accordance with the results from caspase activation (Fig. 4A), no apoptotic nuclear fragmentation can be found for cells deprived of glucose.

4. Discussion

Manipulation of the source or metabolization of carbohydrates influences the pathways of cellular energy metabolism by altering the supply of substrates for glycolysis and the pentose phosphate pathway. By this, changes in the availability of glycolytic substrates will also affect the cell's ability to form NADPH. The latter represents the crucial coupling agent for a cellular mechanism involved in antioxidative defense, the glutathione system, since its regeneration (from GSSG to GSH, catalyzed by glutathione reductase) is dependent on reducing power. These facts motivated us to investigate whether the supply of glucose would influence the cellular ROS quenching mechanisms and whether an increase of the sensitivity towards ROS caused by conditions of glucose deprivation can be causally attributed to impaired GSH regeneration or synthesis. For this purpose, two different metabolic inhibitors were used to (i) deplete total glutathione by inhibition of γ-glutamyl-cystein synthase (an ATP-requiring step within the pathway of GSH de novo synthesis [7,9]; inhibited by BSO [21]) and (ii) inhibit the regeneration of GSH from the oxidized form, GSSG (catalyzed by NADPH-dependent glutathione reductase [7,9]; inhibited by BCNU [22]).

In a first set of experiments, the overall cytotoxicity of PDT under such conditions was analyzed. Both, glucose withdrawal as well as depletion of cellular glutathione (or inhibition of its regeneration) resulted in a significant increase in cytotoxicity. These results are consistent with the findings of Miller et al. [23] where addition of BSO to several cell lines caused increased cytotoxicity of Photofrin PDT and γ irradiation. The reason why BCNU resulted in a less pronounced cytotoxicity when compared to BSO may be caused by an incomplete inhibition of glutathione reductase when BCNU was applied as described; furthermore (see Fig. 2), at the time of PDT, the samples treated with BCNU are still provided with about 5 mM intracellular GSH, which is available for an initial quenching of ROS. Similar results were obtained by another group who studied the effect of glucose deprivation on the cellular sensitivity towards H_2O_2 [10]. The striking similarity between samples withdrawn of glucose as a substrate for the pentose phosphate cycle and those with hindered GSH metabolism give first evidence for a causal relationship between the increased sensitivity of glucose deprived cells and their GSH metabolism.

This suspected causal relationship of carbohydrate metabolism and the GSH system is supported by measurement of intracellular GSH (Fig. 2). Two explanations can be given for the severe reduction of $[GSH]_{ic}$ in glucose-free samples: (i) ROS generated under conditions of increased respiratory activity that compensates for an inhibition of glycolytic ATP production in GF-DMEM samples cause oxidation and reduction of $[GSH]_{ic}$ which, furthermore, cannot be regenerated due to a lack of PPP substrates; and (ii) the detrimental effect of glucose-free conditions on the cellular energy metabolism and ATP generation results in a reduced rate of the ATP-requiring de novo synthesis of GSH. It is not clear to which extent these explanations hold true of the data shown; however, both apply to the central hypothesis of this study.

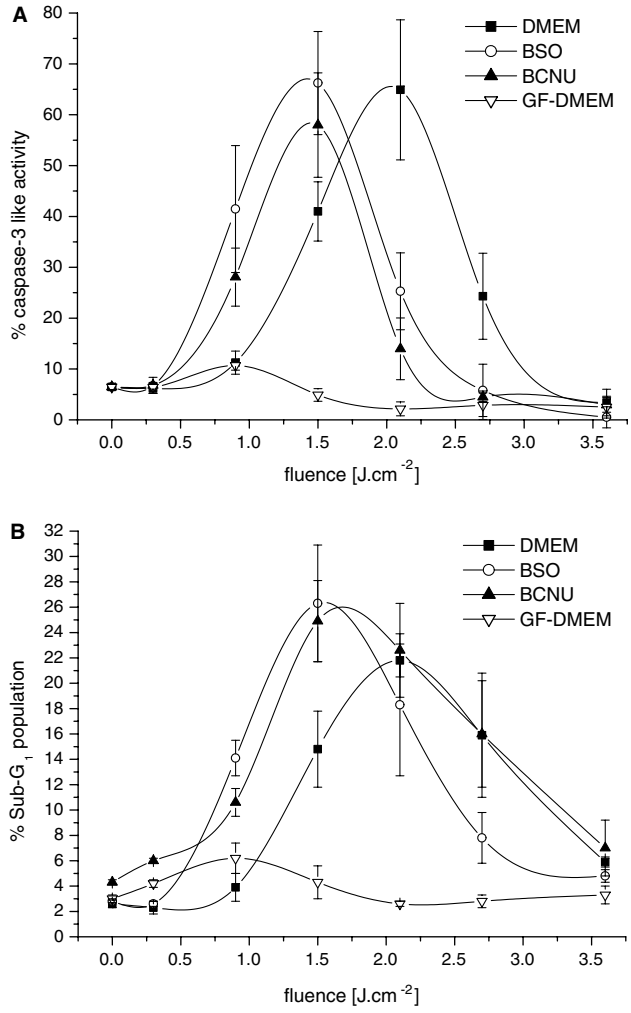

Fig. 4. Apoptosis induced by photodynamic treatment. Photosensitized cells with impaired glucose/glutathione metabolism were analyzed for caspase activation and nuclear fragmentation. When indicated ('BSO'/'BCNU'), 3 mM buthionine sulfoximine was added 18 h and 500 μM 1,3-bis-(2-chloroethyl)-1-nitrosourea was added 1 h before illumination, respectively; GF-DMEM indicates glucose free medium. (A) Activity of caspase-3 like enzymes 5 h p.i. related to UV-treated cells (a homogenous apoptotic cell population). (B) Nuclear fragmentation (Sub-G_1 peak) 8 h p.i. All data are representatives of three independent experiments ± Gaussian error bars (A) or S.E.M. (B).

The results of measurement of cytotoxicity are essentially strengthened by analysis of PDT-based generation of ROS under identical conditions. PDT applied to cells with impaired glucose or GSH metabolism results in increased intracellular ROS levels. When using DCFH-DA for measurement of ROS, it is important to note that this dye does not measure singlet oxygen itself [24], hence DCFH-DA may only give semiquantitative information about ROS generated by PDT but yet allowing meaningful comparison within a fluence series of a given PDT treatment. The high ROS signal of GF-DMEM samples (even without PDT), therefore, could reflect other reactive oxygen species generated by cellular respiration fuelled by pyruvate [2]. Taken together, the effect of glucose deprivation/BSO or BCNU treatment on PDT-based generation of ROS is most obviously indicated by the initial increase/slope of the signal at the transition from 0 to 0.3 J cm^{-2}, which is – regardless of the absolute values – more pronounced for all samples compared to the standard DMEM sample. We have previously shown [16] that massive necrosis occurs under glucose-free conditions at higher light fluences; this is the reason why the ROS signal for GF-DMEM samples even decrease at higher PDT doses, since the dye is most probably released from necrotic cells.

Based on these results, we further analyzed the effect of impaired glutathione metabolism on the appearance and extent of apoptosis. Apoptosis induction could be observed for samples treated with BSO or BCNU, but the apoptotic window was shifted to lower fluences, indicating a higher susceptibility of cells with an impaired GSH system to oxidative stress. The overall extent of apoptotic cells remained unchanged. Interestingly, the shift of the apoptotic window for the BSO and BCNU samples was almost identical contrasting the different increase in cytotoxicity following PDT as shown in Fig. 1. Although BCNU is widely used as a specific inhibitor of glutathione reductase [22], this observation could be linked to the multi-functionality of this drug which may alkylate various cellular targets; thus, apart from inhibition of glutathione reductase another mode of action of BCNU might be involved in rendering cells more susceptible to induction of apoptosis.

In clear contrast, no apoptotic cells were found in samples deprived of glucose. This effect is a result of the lack of ATP in these probes and *not* of a destruction of enzymes required for apoptosis (see BSO and BCNU treated samples which clearly show apoptosis) as it was suggested by a reviewer of our earlier work [16]. For PDT, at least two studies emphasize the importance of glycolytic ATP production to compensate for a reduction of the mitochondrial membrane potential during the apoptotic process [16,25] to maintain high ATP levels after PDT [19]. Taken together, while artificial inhibition of the cell's pathways to synthesize and regenerate GSH causes an increased sensitivity towards ROS and an augmented cytotoxicity of PDT, it does not interfere with the ability to undergo active cell death. In contrast, omission of glucose not only decreases the cell's antioxidative capabilities but also abolishes the energy supply for apoptosis.

Several attempts to influence the cellular antioxidative systems have been published where: (i) systemic administration of BSO augmented photofrin-PDT mediated destruction of intracerebral 9L gliosarcoma [26], (ii) addition of several low molecular weight ROS scavengers (e.g., GSH, L-tryptophan and *N*-acetyl-L-cysteine) exerted a protective effect against PDT damage [27,28], and (iii) protection against PDT induced cell injury was achieved by addition of enzymes involved in the antioxidative systems (e.g., superoxide dismutase, catalase and lipoamide dehydrogenase) [27,29]. Additionally, the possibility of manipulating the cellular antioxidative metabolism by alteration of the concentration or the nature of the carbon source present in the culture medium was examined recently: substitution of glucose by galactose results in an increased rate of cell death induced by hydrogen peroxide [10,12]; an opposite effect was found for hepatocytes when fructose was added as the major carbohydrate in a hypoxia-reoxygenation model [11]. Further evidence comes from studies where: (i) addition of fructose-1,6-bisphosphate protected cortical neurons against oxidative stress partly by increasing glutathione reductase activity [30] and (ii) addition of pantothenic acid/pantothenol (precursors of coenzyme-A) increased net biosynthesis of GSH by boosting cellular energetics [31]. These studies impressively show a causal relationship between carbohydrate-,

energy metabolism and the cell's antioxidative competence as expressed by the glutathione system.

It is important to note that the special case of glucose deprivation causes further consequences besides those mentioned above; among them, alteration of glucose-regulated gene expression or changes in the fluxes of metabolic pathways deriving substrates from glycolysis or the Krebs cycle may be named [32]. However, as demonstrated in this study, changes in metabolic systems which are instantly affected by glucose deprivation (such as cellular energetics and the production of reducing equivalents) and their consequences for the outcome of PDT in vitro readily can be explained without further need of taking into account specific changes in signal transduction or other more global metabolic regulation.

In conclusion, the novelty of the present study goes back to the facts that (i) it provides first evidence that management of the in vitro efficiency of PDT can be achieved not only by external administration of components involved in the antioxidative defense, but also by manipulation of the carbohydrate metabolism aiming at the control of the cellular GSH system and (ii) it also takes into account the possible impact of altered carbohydrate metabolism on the energetics and, in consequence, the mode of cell death.

References

[1] Curtin, J.F., Donovan, M. and Cotter, T.G. (2002) Regulation and measurement of oxidative stress in apoptosis. J. Immunol. Methods 265 (1–2), 49–72.

[2] Skulachev, V.P. (1999) Mitochondrial physiology and pathology: concepts of programmed death of organelles, cells and organisms. Mol. Aspects Med. 20 (3), 139–184.

[3] Skulachev, V.P. (2001) The programmed death phenomena, aging, and the Samurai law of biology. Exp. Gerontol. 36 (7), 995–1024.

[4] Dolmans, D.E., Fukumura, D. and Jain, R.K. (2003) Photodynamic therapy for cancer. Nat. Rev. Cancer 3 (5), 380–387.

[5] Oleinick, N.L., Morris, R.L. and Belichenko, I. (2002) The role of apoptosis in response to photodynamic therapy: what, where, why, and how. Photochem. Photobiol. Sci. 1 (1), 1–21.

[6] Plaetzer, K., Kiesslich, T., Verwanger, T. and Krammer, B. (2003) The modes of cell death induced by PDT: an overview. Med. Laser Appl. 18 (1), 7–19.

[7] Dickinson, D.A. and Forman, H.J. (2002) Cellular glutathione and thiols metabolism. Biochem. Pharmacol. 64 (5–6), 1019–1026.

[8] Lu, S.C. (1999) Regulation of hepatic glutathione synthesis: current concepts and controversies. FASEB J. 13 (10), 1169–1183.

[9] Meister, A. and Anderson, M.E. (1983) Glutathione. Annu. Rev. Biochem. 52, 711–760.

[10] Le Goffe, C., Vallette, G., Charrier, L., Candelon, T., Bou-Hanna, C., Bouhours, J.F. and Laboisse, C.L. (2002) Metabolic control of resistance of human epithelial cells to H_2O_2 and NO stresses. Biochem. J. 364 (Pt 2), 349–359.

[11] Frenzel, J., Richter, J. and Eschrich, K. (2002) Fructose inhibits apoptosis induced by reoxygenation in rat hepatocytes by decreasing reactive oxygen species via stabilization of the glutathione pool. Biochim. Biophys. Acta 1542 (1–3), 82–94.

[12] Le Goffe, C., Vallette, G., Jarry, A., Bou-Hanna, C. and Laboisse, C.L. (1999) The in vitro manipulation of carbohydrate metabolism: a new strategy for deciphering the cellular defence mechanisms against nitric oxide attack. Biochem. J. 344 (Pt 3), 643–648.

[13] Eguchi, Y., Srinivasan, A., Tomaselli, K.J., Shimizu, S. and Tsujimoto, Y. (1999) ATP-dependent steps in apoptotic signal transduction. Cancer Res. 59 (9), 2174–2181.

[14] Yasuhara, N., Eguchi, Y., Tachibana, T., Imamoto, N., Yoneda, Y. and Tsujimoto, Y. (1997) Essential role of active nuclear transport in apoptosis. Genes Cells 2 (1), 55–64.

[15] Kass, G.E., Eriksson, J.E., Weis, M., Orrenius, S. and Chow, S.C. (1996) Chromatin condensation during apoptosis requires ATP. Biochem. J. 318 (Pt 3), 749–752.

[16] Oberdanner, C.B., Kiesslich, T., Krammer, B. and Plaetzer, K. (2002) Glucose is required to maintain high ATP-levels for the energy-utilizing steps during PDT-induced apoptosis. Photochem. Photobiol. 76 (6), 695–703.

[17] Gonzalez, R.J. and Tarloff, J.B. (2001) Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. Toxicol. In Vitro 15 (3), 257–259.

[18] Griffith, O.W. (1983) Glutathione and Glutathione Disulphide (Bergmeyer, H.U., Bergmeyer, J. and Grassl, M., Eds.), Methods of Enzymatic Analysis, VIII, pp. 521–529, Verlag-Chemie, Weinheim.

[19] Plaetzer, K., Kiesslich, T., Krammer, B. and Hammerl, P. (2002) Characterization of the cell death modes and the associated changes in cellular energy supply in response to AlPcS4-PDT. Photochem. Photobiol. Sci. 1 (3), 172–177.

[20] Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65 (1–2), 55–63.

[21] Griffith, O.W. (1982) Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J. Biol. Chem. 257 (22), 13704–13712.

[22] Frischer, H. and Ahmad, T. (1977) Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU [1,3-bis(chloroethyl)-1-nitrosourea]. J. Lab. Clin. Med. 89 (5), 1080–1091.

[23] Miller, A.C. and Henderson, B.W. (1986) The influence of cellular glutathione content on cell survival following photodynamic treatment in vitro. Radiat. Res. 107 (1), 83–94.

[24] Bilski, P., Belanger, A.G. and Chignell, C.F. (2002) Photosensitized oxidation of 2′,7′-dichlorofluorescin: singlet oxygen does not contribute to the formation of fluorescent oxidation product 2′,7′-dichlorofluorescein. Free Radic. Biol. Med. 33 (7), 938–946.

[25] Kirveliene, V., Sadauskaite, A., Kadziauskas, J., Sasnauskiene, S. and Juodka, B. (2003) Correlation of death modes of photosensitized cells with intracellular ATP concentration. FEBS Lett. 553 (1–2), 167–172.

[26] Jiang, F., Lilge, L., Belcuig, M., Singh, G., Grenier, J., Li, Y. and Chopp, M. (1998) Photodynamic therapy using Photofrin in combination with buthionine sulfoximine (BSO) to treat 9L gliosarcoma in rat brain. Lasers Surg. Med. 23 (3), 161–166.

[27] Kliukiene, R., Maroziene, A., Nivinskas, H., Cenas, N., Kirveliene, V. and Juodka, B. (1997) The protective effects of dihydrolipoamide and glutathione against photodynamic damage by Alphtalocyanine tetrasulfonate. Biochem. Mol. Biol. Int. 41 (4), 707–713.

[28] Perotti, C., Casas, A. and Del, C.B.A.M. (2002) Scavengers protection of cells against ALA-based photodynamic therapy-induced damage. Lasers Med. Sci. 17 (4), 222–229.

[29] Hadjur, C., Richard, M.J., Parat, M.O., Favier, A. and Jardon, P. (1995) Photodynamically induced cytotoxicity of hypericin dye on human fibroblast cell line MRC5. J. Photochem. Photobiol. B 27 (2), 139–146.

[30] Vexler, Z.S., Wong, A., Francisco, C., Manabat, C., Christen, S., Tauber, M., Ferriero, D.M. and Gregory, G. (2003) Fructose-1,6-bisphosphate preserves intracellular glutathione and protects cortical neurons against oxidative stress. Brain Res. 960 (1–2), 90–98.

[31] Slyshenkov, V.S., Dymkowska, D. and Wojtczak, L. (2004) Pantothenic acid and pantothenol increase biosynthesis of glutathione by boosting cell energetics. FEBS Lett. 569 (1–3), 169–172.

[32] Foufelle, F., Girard, J. and Ferre, P. (1998) Glucose regulation of gene expression. Curr. Opin. Clin. Nutr. Metab. Care 1 (4), 323–328.