Light-induced Photoactivation of Hypericin Affects the Energy Metabolism of Human Glioma Cells by Inhibiting Hexokinase Bound to Mitochondria

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ABSTRACT

Glucose-dependent energy required for glioma metabolism depends on hexokinase, which is mainly bound to mitochondria. A decrease in intracellular pH leads to a release of hexokinase-binding, which in turn decreases glucose phosphorylation, ATP content, and cell proliferation. Thus, intracellular pH might be a target for therapy of gliomas, and a search for agents able to modulate intracellular pH was initiated. Hypericin, a natural photosensitizer, displays numerous biological activities when exposed to light. Its mechanism and site of action at the cellular level remain unclear, but it probably acts by a type II oxygen-dependent photosensitization mechanism producing singlet oxygen. Hypericin is also able to induce a photogenerated intracellular pH drop, which could constitute an alternative mechanism of hypericin action. In human glioma cells treated for 1 h with 2.5 μg/ml hypericin, light exposure induced a fall in intracellular pH. In these conditions, mitochondria-bound hexokinase was inhibited more than 100-fold and dose-dependent manner, associated with a decreased ATP content, a decrease of mitochondrial transmembrane potential, and a depletion of intracellular glutathione. Hexokinase protein was effectively released from mitochondria, as measured by an ELISA using a specific anti-hexokinase antibody. In addition to decreased glutathione, a response to oxidative stress was confirmed by the concomitant increase in mRNA expression of γ-glutamyl cysteine synthetase, which catalyzes the rate-limiting step in overall glutathione biosynthesis, and is subject to feedback regulation by glutathione. Hypericin also induced a dose- and light-dependent inhibition of [3H]thymidine uptake and induced apoptosis, as demonstrated by annexin V-FITC binding and cell morphology. This study confirmed the mitochondria as a primary target of photodynamic action. The multifaceted action of hypericin involves the alteration of mitochondria-bound hexokinase, initiating a cascade of events that converge to alter the energy metabolism of glioma cells and their survival. In view of the complex mechanism of action of hypericin, further exploration is warranted in a perspective of its clinical application as a potential phototoxic agent in the treatment of glioma tumors.

INTRODUCTION

Much attention has been paid recently to the development of new tools for application in PDT. PDT is based on the use of photosensitizers, which acquire their antitumor activity when excited by light and are relatively inactive in the absence of light. The simultaneous presence of light and photosensitizers in the target tumor is required (1). Photofrin, a porphyrin mixture, is the first PDT agent authorized for the treatment of cancer (2, 3). Photoactivated hematoporphyrin derivatives produced antitumor effects mediated by singlet oxygen as the cytotoxic agent (4, 5) but are not clinically satisfying (6).

Hypericin is a natural photosensitizing diathraquinone, which can be extracted from plants of the Hypericum genus (7). Ingestion of hypericin-containing plants by grazing animals has been shown to cause hypericism, a condition of severe sensitivity to light (8). A renewed interest in hypericin as a photosensitizer has recently arisen because the compound displays a potent antitretroviral activity that is enhanced more than 100-fold in the presence of light (9). When exposed to light (600 nm), photoactivated hypericin inhibits mitochondrial succinoxidase (10), an in vitro enzymatic model for the screening of photochemotherapeutics. Several in vitro photosensitizing effects have been reported, such as oxidation of lipids, amino acids, and proteins, disruption of membrane function (7), DNA interactions (11), inhibition of mitochondrial function (12), protein kinase C (13), epidermal growth factor receptor, and tyrosine kinase activity (14) and inhibition of proliferation (15). In response to various photosensitizer-mediated PDT, cells undergo apoptosis mediated by various pathways (16–18) in a Bcl-2- and p53-independent manner (19).

An in vivo antitumor effect of photoactivated hypericin has also been demonstrated (20).

Most evidence suggests that, after excitation by visible light, hypericin undergoes a type II oxygen-dependent mechanism in which energy transfer from excited triplet state hypericin to ground state generates singlet oxygen and O2 as the primary oxidizers (21), although a type I mechanism (involving radical formation) is also possible in some cases (14). It was shown that photoactivated hypericin was able to produce a photogenerated pH drop through an intramolecular proton transfer in the exited state of the molecule, which is likely to proceed solvent acidification (22), and an pHi decrease has been demonstrated in living cells exposed to light with hypericin (23). It was postulated that this effect is one of the possible processes responsible for the photosensitizing properties of hypericin involved in virucidal and antitumor activities (23). Hypericin also has biological properties in the dark, such as potent antidepressant (24) and antiretroviral activities (9). In addition, hypericin does not show genotoxicity or toxicity in animals and humans and could therefore be used clinically as a safe photodynamic antitumor agent (6).

Glioblastoma multiforme, the most common primary brain tumor in humans, is almost always fatal (25). Treatment consists of surgery, radiation and/or chemotherapy (26). The dismal prognosis has stimulated the search for new therapies. PDT has recently come to the forefront as an adjuvant to surgery for the treatment of intracranial tumors, primarily because of its tumor selectivity (27). Therefore, PDT offers a more selective approach to the treatment of brain malignancies compared with other standard available treatment modalities and is similar to the targeted approaches that are presently under investigation (28).

The metabolism of gliomas, as that of many tumors, is characterized by a high rate of aerobic glycolysis (29, 30). The reduction of the glycolytic pathway by energy deprivation leads to a growth inhibition of glioma cells (31). Glucose-dependent energy required for glioma metabolism is regulated by HK (ATP:α-hexose 6-phosphotransferase;
EC 2.7.1.1), which catalyzes the phosphorylation of glucose for its entry into glycolysis. HK is bound to porins located at the outer mitochondrial membrane (32), which form a complex containing the PBR and ATP-ADP translocase (33). Increased density of PBRs in glioblastoma was correlated with glucose utilization and aggressiveness (34).

pHii has been shown to be a critical regulator of HK-mitochondria interactions and thus of glycolysis. An induced intracellular acidification for 5 min was sufficient to decrease the activity of mHK, whereas glycolysis parameters (ATP pool, glucose 6-phosphate, lactate-pyruvate ratio) were unaffected (35). We showed previously that lonidamine (a glycolysis inhibitor) reduced growth rate of human gliomas in vitro and in vivo and induced a pH drop (36). Because all cellular processes may be affected by the pHii, the variations of which seem to be important in controlling the cell cycle and the proliferative capacity of cells (37), the pHii might be a target for therapy of gliomas, and a search for agents able to modulate pHii was initiated.

The mitochondrion has been proposed as a primary target of photodynamic action (38, 39). Very recently, the therapeutic potencies of porphyrins used as PDT agents were shown to correlate closely with both densities and affinities for PBRs, and this suggests that the action of porphyrin PDT is mediated by PBRs (40). Hematoporphyrin derivatives have also been shown to inhibit mitochondrial enzymes and to deplete cellular ATP in a light dose-dependent manner (41). Numerous studies have demonstrated recently that cells undergoing apoptosis exhibit a decrease in the Δψm preceding nuclear signs of apoptosis, suggesting that early alterations of mitochondrial function may be important for the apoptotic process (42–46).

This study was designed to evaluate the potential effect of photostimulated hypericin in living glioma cells on pHii in relation to HK activity and subcellular distribution, the ATP pool, [3H]PK11195 uptake, oxidative stress, Δψm, mitochondrial ultrastructure, cell proliferation, and apoptosis.

MATERIALS AND METHODS

Reagents. Hypericin was obtained from Carl Roth (Lauterbourg, France). [3H]Thymidine (20 Ci/ml) was obtained from Dupont/NEN (Les Ulis, France). DMEM, L-glutamine, PBS, trypsin-EDTA, and penicillin-streptomycin were obtained from Sigma Chemical Co. (St. Quentin Fallavier, France). FCS was obtained from Dutschner (Brumath, France).

Cell Culture and Treatments. The human glioblastoma cell line SNB-19 (47) was grown in DMEM containing 25 mM glucose at pH 7.4 and supplemented with 10% FCS, 2 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cells were maintained at 37°C, with 5% CO₂/95% air humidified incubator, and harvested for passage when they reached confluence. For experiments, cells were collected from subconfluent monolayers with 0.5 mg/ml trypsin and EDTA (0.2 mg/ml). Cell viability was estimated by trypan blue exclusion and was >95% before each experiment. Cells were plated in their usual medium and allowed to attach overnight. Hypericin was dissolved in DMSO at 3 mg/ml, and the final concentration of DMSO in cells was 0.2%. In all of the experiments, cells were washed once with PBS (150 mM NaCl, 150 mM sodium phosphate, pH 7.4) and then incubated with the desired concentration of hypericin for 1 h in the dark prior to light exposure. Control wells were sham-exposed to light.

Light Exposure. Because coherent laser light is not necessary for PDT (48), a simple wide emission spectrum light source was used. Cells in dishes were placed 20 cm under a 75 W tungsten lamp. The light dose was measured by integrating the energy signal in W/cm² over the entire period of light exposure with an YSI-Kettering Model 65A radiometer. The light dose was extinguished when the total light dose reached 1.5 J/cm² (2 min of light exposure) or 7.5 J/cm² (10 min of light exposure). Subbed light conditions were achieved by reducing the sources of illumination to a minimum and by protecting the samples from light with aluminum sheets.

Hypericin Intracellular Accumulation Studies. Cells were grown to confluence in 35-mm culture dishes in standard culture conditions. The dye was added at a final concentration of 0.5 and 2.5 µg/ml to the dishes seeded with 10⁵ cells. After varying incubation times, the cells were washed twice with PBS, and the hypericin taken up by the cells was extracted by adding 1.5 ml of PBS containing 0.1% Triton X-100 for 15 min. The medium containing the extracted hypericin was transferred to the cuvette of a fluorometer. The hypericin concentration was determined by a fluorometric technique from the intensity of emission peak at 599 nm under excitation at 337 nm, with a standard curve generated from 0.1% Triton solutions containing known concentrations of hypericin (49).

[3H]Thymidine Uptake Assay. [3H]Thymidine uptake assay in SNB-19 glioma cells was performed as described (36). Control radioactivity was expressed as 100%, and assays were expressed as percentage of control.

Cytofluorimetric Analysis of Δψm. To evaluate the Δψm, cells (5 × 10⁵) cultivated in 35-mm tissue culture dishes were incubated with DIOC₆(3) (Molecular Probes, Eugene, OR; 40 nm in PBS) for 15 min at 37°C. The cells were then rinsed twice with PBS, trypsinized, and analyzed by flow cytofluorometry. Treatment of cells with 100 µM mCICCP (15 min; Sigma) served as control for staining with DIOC₆(3). In all experiments, forward and side scatters were gated on the normal-sized, viable cells. The cytofluorometer was set to measure forward and side scatters, DIOC₆(3) fluorescence (FL1, 525-nm band-pass), and hypericin fluorescence (FL3, 630-nm band-pass).

Electron Microscopy. Cells cultivated in 35-mm tissue culture dishes were fixed with 2% glutaraldehyde in serum-free culture medium and with a mixture of cacodylate (0.2 M; pH 7.4) and 4% glutaraldehyde (1:1). After three washes with cacodylate and 0.4 M saccharose (1:1), cells were post-fixed with cacodylate (0.3 M; pH 7.4) and 2% osmium tetroxide (1:1) and dehydrated in a graded ethanol series. Cells were collected and included in “Epon” resin by usual techniques. Sections of cells were stained with uranyl acetate and lead citrate, transferred to grids, and observed with a Zeiss EMCR 10 microscope.

Annexin V-FITC Labeling. Apoptosis was detected and quantified in cells (5 × 10⁵) by staining with annexin V-FITC (which measures the aberrant phosphatidylserine exposure on the outer plasma membrane leaflet; Euromedex, Souffelweyersheim, France; 1.8 µg/ml in 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) for 15 min in the dark at room temperature and analyzed by flow cytofluorometry. The cytofluorometer was set to measure forward and side scatters, annexin V-FITC fluorescence (FL1, 525-nm band-pass), and hypericin fluorescence (FL3, 630-nm band-pass). Plasma membrane integrity was estimated by trypan blue exclusion.

Preparation of HK Subcellular Fractions, Activity Assay, and Immunodetection. The subcellular fractionation and enzyme activity were performed as published previously (35). Immunodetection of HK protein was performed by ELISA using a rabbit polyclonal anti-type I human HK antibody (provided by Dr. Citro, Rome, Italy) and previously characterized for specificity (50). ELISA conditions were as described (50), and the anti-HK antibody was used at the dilution of 1:200 in ELISA plates coated with 5 µg of protein from the mHK fractions issued from hypericin-treated cells. The antibody binding was detected by incubation with peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma; 1:2000 dilution) and was revealed using the o-phenylenediamine and hydrogen peroxide reagents.

ATP Determination. The ATP pool was determined with the ATP bioluminescent assay kit (Sigma) by means of the luciferin-luciferase reaction (35) using a Lumac Biocounter M1500 luminometer (Lumac bv, Landgraaf, the Netherlands).

Binding of [3H]PK11195. Binding assays were performed on cell cultures as described previously (51) with slight modifications. Briefly, the medium of cells in exponential phase growth in 60-mm Petri dishes was replaced by 140 mM NaCl buffered with 10 mM NaH₂PO₄ (pH 7.4, 37°C), 2.6 mM KCl, and 1.4 mM KH₂PO₄ for 10 min to remove binding inhibitors that might be present in the medium. The washing buffer was replaced with buffer containing [3H]PK11195. Non-specific binding was determined in the presence of 10 µM unlabeled PK11195. After incubation (20 min at 37°C), the ligand mixture was discarded by aspiration, and labeled monolayers were washed with cold PBS. Cells were dissolved in 1 ml 0.2 M NaOH and placed into vials with 10 ml of scintillant liquid and assayed for tritium by liquid scintillation spectroscopy at 60% efficiency. Each sample was performed in triplicate. Binding studies were carried out using 1 nm [3H]PK11195 in the presence or absence of unlabeled PK11195.

Laser Microspectrofluorometry of Snarf-1-AM-stained Cells. After 45-min incubation with hypericin in the dark, cells were loaded with Snarf-1-AM

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(Dr. James D. Miller, Ph.D.)

Intracellular GSH Level and mRNA Expression for γ-GCS. Total GSH, determined by the enzymatic recycling assay, was extracted from adherent cells with 20% sulfo-5-salicylic acid as described (54, 55). mRNA expression of γ-GCS was measured by a reverse transcription-PCR-based method as described (56) with total RNA isolated from living cells using Trizol Reagent (Life Technologies, Inc., Cergy Pontoise, France) according to the manufacturer’s instructions. cDNA was prepared in a oligo(dT)-primed reverse transcriptase reaction consisting in 2 μg of total RNA; a total volume of 20 μl of cDNA (1 μl) was then used for PCR amplification in a final volume of 50 μl for γ-GCS and β2-microglobulin. β2-Microglobulin PCR was performed using the following primers: sense, 5'-CATCCAGCGTACTCAAAAGA-3'; and antisense, 5'-GACAAGTCTGAATGCTCCAC-3'. Thermal cycling was as follows: denaturation at 94°C for 1 min, annealing and extension at 72°C for 2 min, 38 cycles for γ-GCS PCR; and denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, 25 cycles for β2-microglobulin PCR. The mRNA of β2-microglobulin, used as an internal standard, was measured concurrently with that of γ-GCS from the same RNA extracts. PCR products (6 μl) were separated by electrophoresis in a 1.8% agarose gel, visualized by ethidium bromide (0.5 μg/ml) staining under UV exposure for 6/30-s exposure. The image was then processed by software and analyzed using the NIH 1.60 version image program and photographed. The linear ranges of amplification for β2-microglobulin cDNA were established by varying the number of cycles.

Statistical Analysis. The Mann-Whitney U test was used to assess the effects of photoactivated hypericin on HK activity and immunodetection and on the ATP pool. P values were considered to be significant if they were ≤0.05.

RESULTS

Hypericin Uptake Studies. Kinetics of hypericin uptake by SNB-19 cells under standard conditions showed that hypericin uptake reached a plateau after ~60 min (Fig. 1). Maximal dye accumulation was ~900 ng/106 cells and 200 ng/106 cells at the concentration of 0.5 and 2.5 μg/ml, respectively. The rate of uptake of hypericin into the cell line was the same for the two different drug concentrations. For all subsequent experiments, cells were incubated with hypericin for 1 h before light exposure.

Effect of Hypericin on pH. Hypericin-induced pH changes were monitored with Snarf-1, a specific pH probe, by measurements of the If of two bands (635 and 592 nm) of its emission spectrum and after calculation of the ratio: R = If635/If592. A calibration curve giving an estimate of pH values is presented in Fig. 2A. The mean R, obtained from the analysis of 20 cells, increased from 0.48 to 0.77 at pH 6.0 and pH 8.0, respectively. Fig. 2, B–E, shows the distributions of pH values in HEPE-S saline buffer (pH 7.4) registered in hypericin-treated cells (2.5 μg/ml; 1 h) and untreated control cells before and after 2 and 10 min of light exposure (1.5 and 7.5 J/cm2, respectively). The mean R, obtained from the analysis of 20 cells, decreased from 0.60, corresponding to a pH of 7.2–7.3 in control cells (Fig. 2B) to 0.58 (pH 7.1–7.2) and 0.52 (pH 6.4–6.6) of hypericin-treated cells upon light exposure at 1.5 J/cm2 (Fig. 2D) and 7.5 J/cm2 (Fig. 2E), respectively. The R obtained in cells treated with hypericin in subdued light conditions (Fig. 2C), corresponding to the minimum of light in the laboratory, was similar to that obtained in control cells. No modification of pH was registered in cells exposed to light treated with 0.5 μg/ml hypericin (data not shown).

Effect of Hypericin on Subcellular Distribution of HK Activity and Immunodetection. Cells, hypericin-treated (2.5 and 0.5 μg/ml; 1 h at 37°C) or not treated, were exposed to light (1.5 and 2.5 J/cm2) and not or then were subjected to fractionation. HK activity in the subcellular fractions was measured spectrophotometrically using the glucose 6-phosphate dehydrogenase reaction. In subdued light conditions, mHK was not affected by 0.5 μg/ml hypericin and slightly decreased with 2.5 μg/ml hypericin (~20% decreased activity; Fig. 3A). However, after light exposure, mHK activity was strongly decreased in cells treated with both doses of hypericin in a light-dependent manner. Concomitantly, cHK activity increased in cells exposed to light treated with 0.5 μg/ml hypericin (Fig. 3B) in a light-dependent manner. With 2.5 μg/ml hypericin and in the dark, cHK activity increased 1.3-fold compared with untreated cells, whereas a more modest increase was observed when cells were exposed to the light dose of 7.5 J/cm2. As shown in Fig. 3C, a light-dependent decrease of HK immunodetection, performed by ELISA using a anti-type I human HK antibody, was demonstrated in mHK fractions extracted from cells treated and exposed to light with 2.5 μg/ml hypericin, as compared with untreated control cells. To a lesser degree, cells treated with 0.5 μg/ml photoactivated hypericin also demonstrated a decrease in HK immunodetection.

Effect of Hypericin on ATP Pool. Intracellular ATP was measured after light exposure of cells treated with hypericin, 0.5 and 2.5 μg/ml for 1 h in the dark, in the same samples studied for the subcellular distribution of HK. As shown in Fig. 4, the cells exposed to light treated with 2.5 μg/ml hypericin induced a light-dependent depletion of intracellular ATP pool, as compared with control cells or to cells not exposed to light with hypericin; decreases of 40% and 60% of control values were obtained in cells treated with photoactivated hypericin at the light doses of 1.5 and 7.5 J/cm2, respectively. No decrease in intracellular ATP pool was registered in cells exposed to light treated with 0.5 μg/ml hypericin as compared with controls (Fig. 4).

Effect of Hypericin on [3H]PK11195 Binding to PBRs. [3H]PK11195 binding was measured in cells after various postlight exposure periods with 2.5 μg/ml hypericin for 1 h in the dark. A decrease of the [3H]PK11195-specific binding of 25 and 40% was measured after 4 and 6 h postlight exposure, respectively, in cells treated with hypericin, as compared with cells sham-exposed to light with or without hypericin.
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Fig. 2. Effect of hypericin with or without light exposure on pH. pH was measured after staining of the cells with Snarf-1-AM, a rhodamine derivative that generates two pH-sensitive hands in its fluorescence emission spectrum, thereby enabling pH determination by microspectrofluorometry. In A, the calibration curve was performed by equilibrating pH using nigericin and valinomycin in a high K⁺ buffer previously adjusted to different pH, enabling a rapid equilibration of pHo and pHi. Values are means obtained from the analysis of 20 cells; bars, SD. Cells, treated (C, D, E) or not treated (B) with hypericin (2.5 μg/ml; 1 h in the dark at 37°C), were exposed to the light doses of 1.5 J/cm² (D) and 7.5 J/cm² (E) or not exposed to light (C) before staining with the Snarf-1 fluorescent probe (5 μM; 15 min for 37°C). The Rs were obtained from the analysis of 20 cells. A representative experiment is shown, and the experiment was repeated twice.

Effect of Hypericin on Δψₘ. The changes in Δψₘ were monitored by the potential-sensitive cyanine dye DiOC₆(3). The cells were treated for 1 h in the dark with 2.5 μg/ml hypericin and then exposed to light or not. Cells were immediately stained with DiOC₆(3) (40 nm; 15 min in the dark), trypsinized, and analyzed by flow cytometry. As shown in Fig. 5A (left panel), photoactivated hypericin induced a disruption of the Δψₘ as soon as 30 min postlight exposure incubation [corresponding to DiOC₆(3) incubation and cell preparation for flow cytometry], and thus displayed a reduced average uptake of DiOC₆(3) by 2.3- and 1.4-fold in hypericin-treated cells exposed to the light dose of 7.5 and 1.5 J/cm², respectively, as compared with control cells. Without light exposure, no variation of the Δψₘ was observed in hypericin-treated cells, as compared with cells exposed to light or to control cells not exposed to light (data not shown). Hypericin uptake was similar in each experiment, as verified using its own fluorescence simultaneously recorded in FL3 (representative experiment; Fig. 5A, right lower panel). FL3 fluorescence was also recorded in cells without hypericin and was used as control (Fig. 5A, right upper panel). As a control experiment of DiOC₆(3) uptake, cells were incubated with the protonophore mCCCP (50 μM; 15 min), a substance that dissipates Δψₘ and a 5-fold decrease of the average uptake of the DiOC₆(3) dye was achieved (Fig. 5B).

Effect of Hypericin on Mitochondrial Ultrastructure. Mitochondrial ultrastructure changes were identified in glioma cells by electron microscopy. In control cells without hypericin and in cells with hypericin without light exposure, the mitochondria were rod shaped, with organized cristae, a well-defined mitochondrial membrane, and a finely granular matrix (Fig. 6A). Immediately after light exposure of cells treated with hypericin, a spectrum of degenerative changes was noted in mitochondria. This pattern is characterized by loss of matrix density, mitochondrial swelling with interruption of the mitochondrial membrane, and thin and disorganized cristae, which were sometimes absent (Fig. 6B). The number of mitochondria affected by the treatment was increasing with time postlight exposure and displayed the same pattern of ultrastructural changes (data not shown).

Effect of Hypericin on [³H]Thymidine Uptake. To investigate the effect of photoactivated hypericin on DNA synthesis, human glioma cells were incubated for 1 h with hypericin in the dark, subjected to 2 min (1.5 J/cm²) and 10 min (7.5 J/cm²) of light exposure, and then incubated for 24 and 72 h in fresh medium in the dark. Effects on cell proliferation were assessed using the [³H]thymidine uptake assay. [³H]Thymidine uptake decreased in a dose- and light-related manner (Fig. 7). After a postlight incubation of 24 h (Fig.
with IC₅₀'s >10 µg/ml in subdued light conditions. With 2.5 µg/ml hypericin, a postlight incubation of 72 h produced a 2.5- and 1.6-fold decrease in [³H]thymidine uptake, as compared with a postlight incubation of 24 h at the light dose of 7.5 and 1.5 J/cm², respectively.

Fig. 4. Effect of hypericin with or without light exposure on the ATP pool. ATP was measured in the same cytosolic fraction of Fig. 3 by bioluminescence using the luciferin-luciferase reaction. Results are expressed as the percentage of the ATP content in controls (regarded as being 100%). Columns, mean (n = 3); bars, SE. *P < 0.05 versus cells not exposed to light with hypericin.

7A), the cells exhibited IC₅₀'s >10 µg/ml in subdued light conditions, as compared with 6 and 2 µg/ml at the light doses of 1.5 and 7.5 J/cm², respectively. After a postlight incubation of 72 h (Fig. 7B), a greater decrease in [³H]thymidine uptake was observed with IC₅₀'s of 3 and 1.5 µg/ml at the light doses of 1.5 and 7.5 J/cm², respectively.

Fig. 5. Effect of hypericin with or without light exposure on Δψₘ. Cells, exposed to light or not, with hypericin (2.5 µg/ml; 1 h in the dark) were then stained with the potential-sensitive cyanine dye DiOC₆(3) (40 nm; 15 min in the dark) to monitor Δψₘ alterations. Cells were trypsinized and analyzed by flow cytometry. A, fluorescence of DiOC₆(3) and hypericin (right lower panel) were recorded in the FL1 and FL3 band-pass, respectively; FL3 fluorescence for control cells is indicated in the right upper panel. B, control experiment of DiOC₆(3) uptake performed with the protonophore mClCCP (50 µm; 15 min), a substance that dissipates the Δψₘ. A representative experiment is shown, and the experiment was repeated at least twice.
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Fig. 6. Effect of hypericin with or without light exposure on mitochondria ultrastructure. Cells treated with hypericin (2.5 μg/ml; 1 h in the dark at 37°C) with or without light exposure (7.5 J/cm²) were immediately fixed, stained, and processed for electron microscopy analysis. A, electron microscopy images of glioma cells treated with hypericin in the dark, ×16,000. B, electron microscopy images of cells exposed to light with hypericin illustrating the changes in mitochondrial ultrastructure. ×10,000. Bar, 1 μm.

A postlight incubation of 24 h of cells with 0.5 μg/ml hypericin did not affect the [³H]thymidine uptake, whereas a postlight incubation of 72 h decreased the [³H]thymidine uptake by 10–20%. The decrease in [³H]thymidine uptake induced by photoactivated hypericin was irreversible because after a postlight incubation of 72 h, no cell proliferation occurred.

Effect of Hypericin on the Induction of Apoptosis. Control cells, or cells treated with hypericin (2.5 μg/ml; 1 h in the dark), were exposed to light (7.5 J/cm²). Cells were then incubated in the dark at 37°C for the indicated postlight exposure periods, trypsinized, stained with annexin V-FITC (which measures the aberrant phosphatidylserine exposure on the outer plasma membrane leaflet characteristic of apoptotic cells), and analyzed by flow cytometry. As shown in Fig. 8A, 4% (histogram 3), 14% (histogram 4), 32% (histogram 5), and 88% (histogram 6) of the hypericin-treated cells were found to be annexin V positive (using the Kolmogorov-Smirnov statistics) after a postlight incubation of 3, 4, 5, and 24 h, respectively. The results were compared with control cells (histogram 1) or to cells treated with hypericin without light exposure (histogram 2), where no annexin V-positive cells were found after 24 h. No annexin V-FITC binding was observed in hypericin-treated cells after a postlight incubation of 1 and 2 h (data not shown). In most cases, these cells retained a well-defined plasma membrane in agreement with trypan blue exclusion until 4–5 h after light exposure and was permeable to trypan blue 24 h after light exposure. Characteristic feature of cells undergoing apoptosis was confirmed by electron microscopy analysis of cells treated with photoactivated hypericin. As soon as 2 h after light exposure of cells treated with hypericin, apoptotic events were present, as peripheral cytoplasmic budding (Fig. 8B), peripheral chromatin condensation (Fig. 8C), and loss of nuclear membrane (Fig. 8C).

Effect of Hypericin on Total GSH Level and mRNA Expression for γ-GCS. Total intracellular GSH, of prime importance as a cellular antioxidant, and γ-GCS mRNA expression, which is subject to feedback regulation by GSH, were measured in cells exposed to light or not treated with hypericin. As shown in Fig. 9A, a 2.5-fold decrease of the total intracellular GSH was observed 1 and 2 h after light exposure of cells treated with 2.5 μg/ml hypericin at the light dose of

[Graphs and tables are included here showing data on cell proliferation and GSH levels.]
Fig. 8. Effect of hypericin with or without light exposure on apoptosis. Control cells or hypericin-treated cells (2.5 µg/ml; 1 h in the dark) were subjected to light exposure (7.5 J/cm²). Cells were then incubated in the dark at 37°C for the indicated periods, trypsinized, and stained with annexin V-FITC and analyzed by flow cytometry or processed for electron microscopy analysis. A, annexin V-positive cells 3, 4, 5, and 24 h after light incubation are represented as histogram numbers 3, 4, 5, and 6, respectively. Results were compared with control cells (histogram 1) or to cells treated with hypericin without light exposure (histogram 2) for 24 h. A representative experiment is shown, and the experiment was repeated at least twice. B and C, electron microscopic images illustrating hypericin-treated cells 2 h after PDT undergoing apoptosis: peripheral cytoplasmic budding (B; X5000), peripheral chromatin condensation and loss of nuclear membrane (C; X10,000). Bar, 1 µm.

The effect of photoactivated hypericin on mitochondrial energy and glycolysis at the cellular level has not received much attention until now. As demonstrated previously and in this study, hypericin is able to induce in viable cells a photogenerated pH drop of about 0.5 pH unit (23). On the basis of our previous results on the pH sensitivity of the subcellular distribution of HK activity, we decided to investigate the effect of hypericin on HK distribution and on mitochondrial metabolism. The present experiments demonstrated that in addition to the previously reported inhibition of membrane-bound enzymes, such as succinocinase and protein kinase C (10, 13), hypericin inhibited mHK in living cells in a concentration- and light-dependent manner, although an inhibition in subdued light conditions was also observed at the dose of 2.5 µg/ml. A light-dependent decrease of pH was demonstrated here only at the higher dose (2.5 µg/ml) of hypericin. Thus, the effect on mHK observed in both subdued light conditions with the higher dose of hypericin and with the lower dose (0.5 µg/ml) of photoactivated hypericin cannot be attributed directly to its effect on pH. Nevertheless, acidification could enhance the oxidative damage and should be considered as a mechanism that could help to explain the complex phototoxic properties of hypericin (23).

Although being primarily located in the cell membrane after a short-term incubation, the lipophilic hypericin reaches the cytoplasm, and after a long-term incubation, it reaches the inside of the cell nucleus (11). Another study shows the highest hypericin concentration in the perinuclear region (58). Photoactivated hypericin generates singlet oxygen at a high quantum yield in comparison with other photosensitizers (6), and its membrane perturbation could indirectly
The mitochondria have been proposed as a primary target of photodynamic effects. Total GSH assay was performed on the intracellular content extracted from adherent cells with 20% sulfo-5-salicylic acid. GSH content of nontreated cells was measured after the indicated postlight periods. mRNA-specific amplification product bands for y-GCS (270 bp) and β2-microglobulin (163 bp), used as internal standard, are indicated. A representative experiment is shown, and the experiment was repeated twice. In A, total intracellular GSH was measured after the indicated postlight periods of cells treated with hypericin. Total GSH assay was performed on the intracellular content extracted from adherent cells with 20% sulfo-5-salicylic acid. GSH content of nontreated cells was expressed as 1 (100%), and assays were expressed as GSH treated:GSH control ratio. A representative experiment is shown, and the experiment was repeated twice. In B, y-GCS mRNA expression was performed by reverse transcription-PCR in total RNA isolated from living cells exposed to light (C: 7.5 J/cm²) with hypericin for the indicated postlight periods. mRNA-specific amplification product bands for y-GCS (270 bp) and β2-microglobulin, used as internal standard, are indicated. A representative experiment is shown, and the experiment was repeated at least twice.

The mitochondria have been proposed as a primary target of photodynamic lipid peroxidation caused by \( \text{O}_2^- \) attack (59). Isolated cHK activity was not inhibited by photoactivated hypericin (data not shown), and this confirms that the toxic effects produced by hypericin occur essentially in a membrane environment. HK is bound to porins (32) and forms a complex containing the PBR located on the outer mitochondrial membrane and ATP-ADP translocase on the inner membrane (33). Photoactivated hypericin was found to inhibit the binding of \( ^{[3}P \)PK11195 to PBRs only 4–6 h after light exposure, and this could be a consequence of the cellular toxic effect of photoactivated hypericin. This mitochondrial protein complex probably requires membrane fluidity or catalytic activity, and membrane lipid peroxidation may also contribute to decrease enzyme and receptor functions.

Numerous studies have demonstrated recently that cells undergoing apoptosis exhibit a decrease in \( \Delta \psi_m \), preceding nuclear signs of apoptosis, suggesting that early alterations of mitochondrial function may be important for the apoptotic process (44, 45). A rapid (30 min after light exposure) light dose-dependent decrease in \( \Delta \psi_m \) was observed only with the higher dose of hypericin. As demonstrated in numerous studies, hypericin is able to induce cell death by apoptosis (18) in a Bcl-2- and p53-independent manner (19). The induction of apoptosis was demonstrated here 2 and 4 h after light exposure of cells treated with the higher dose of hypericin by annexin V-FITC binding and cell morphology, respectively, whereas the lower dose failed to induce \( \Delta \psi_m \) decrease and apoptosis.

Indirect evidence of cellular oxidative damage generated by photoactivated hypericin was investigated by measuring the total GSH content and y-GCS mRNA expression. A bell-shaped response of the cellular GSH was observed showing a strong decrease of the total GSH content in the first 2 h after photoactivation of cells treated with the higher dose of hypericin and was followed 1 h later by an important increase. The role of GSH in hypericin phototoxicity is a matter of debate. Recent studies showed that toxicity induced by photoactivated hypericin was unaffected by free radical scavengers, N-acetylcysteine, superoxide dismutase (19), or a GSH-depleting agent, buthionine sulfoximine (58), suggesting that GSH status and free radical generation are not determining factors in hypericin phototoxicity. Our results are supportive of previous studies that have demonstrated the ability of photoactivated hypericin to induce oxidative stress and reduce GSH at the cellular level (62, 63). In a very recent study, Johnson and Pardini (64) showed that GSH reductase activity was associated with hypericin exposure and contributed to its overall toxicity. It should be stressed that our data on the GSH content were obtained using the enzymatic recycling assay, which does not discriminate between reduced GSH and oxidized glutathione. In spite of this limitation, this method was preferred to the monochlorobimane assay. This GSH-specific assay requires a reaction catalyzed by glutathione S-transferase, which needs to be fully functional (65). The phototoxic effects of hypericin could potentially alter the glutathione S-transferase activity and then the GSH determination. The interpretations of our results on the GSH content must take into account the limitation of the enzymatic recycling assay.

Successful antioxidant defense against reactive oxygen species requires a balanced increase in antioxidant enzymes, not only to cope with the radicals initially produced but also the potentially more toxic products resulting from the catalyzed and spontaneous reduction reactions (66). Cellular GSH is synthesized by y-GCS and GSH synthetase. The first reaction catalyzed by y-GCS is a rate-limiting step in overall GSH biosynthesis and is subjected to feedback regulation by GSH (66). After buthionine sulfoximine treatment, the expression of y-GCS increased, with a time course that mirrored the fall in GSH (67). In our study, the increase and/or stabilization of y-GCS mRNA expression observed after light exposure of cells with hypericin occurred at the same time as GSH decreased and seemed to precede the increase of cellular GSH that was measured 3 h after light exposure and confirmed the influence of GSH concentrations on the expression of the gene responsible for its maintenance and its implication in hypericin phototoxicity. There is evidence demonstrating that the intracellular redox state regulates a spectrum of genes in response to oxidative stress induced by the higher dose of hypericin (68). The transcriptional induction of the y-GCS gene plays an important role in adaptation to quinone-induced oxidative stress, leading to increased y-GCS activity and de novo GSH synthesis. We were unable to demonstrate a decrease in GSH content in the first 2 h after light exposure with the lower dose of hypericin, but a similar increase was noted after a longer postlight exposure period (5 h) as that occurring with the higher dose of hypericin. This suggests that the oxidative stress induced by the higher dose of hypericin exceeds the antioxidant defenses of SNB-19 cells, whereas these defenses are sufficient to repair the damages caused by the lower dose of the photosensitizer. Despite this cellular response, the cells exposed to light with the high dose of hypericin induced irreversible inhibition of...
DNA synthesis, whereas the lower dose only slightly affected cell proliferation (10–20%).

Numerous studies have demonstrated the central role of mitochondria in apoptosis initiation (45) and as a primary target of photodynamic action (38, 40, 60). The porin-PBR-translocator complex may constitute the permeability transition pore, and the physiological existence of kinase porin translocator complexes at the mitochondrial surface has been demonstrated (68, 69). Kinase complex formation may serve regulatory functions, such as regulation of the kinase activity, stimulation of oxidative phosphorylation, regulation of the permeability transition pore, and finally regulation of apoptosis. Our study confirms that mitochondria are an important target of PDT and emphasizes that mHK, which initiates and regulates the glycolytic flux, constitutes one of the main targets of photoactivated hypericin and could be responsible for the progression of toxicity from a subcellular to a cellular level. Treatment targeting mitochondria-dependent functions leading to the promotion of apoptosis might improve the therapy of gliomas. Hypericin does not show genotoxicity or toxicity in animals, even at high doses (20, 57). In humans, further studies are needed to determine the safety and efficacy of hypericin in the long term. The clinical potential of hypericin as a phototoxic agent in vivo for the treatment of gliomas will depend on its pharmacokinetics, i.e., its capacity to accumulate in the tumor in significant concentrations, when administered at subtoxic doses.

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