Molecular aspects of photodynamic therapy: low energy pre-sensitization of hypericin-loaded human endometrial carcinoma cells enhances photo-tolerance, alters gene expression and affects the cell cycle

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Abstract Photosensitization of HEC1-B cells with a low concentration of hypericin and doses of light below 10 J/cm^2 caused cell death (apoptosis occurred mainly at doses between 2 and 5 J/cm^2, whereas necrosis prevailed above 6 J/cm^2). However, pre-exposure of cells to innocuous irradiation (2 J/cm^2) and successive challenge with a light dose that normally induced apoptosis (5 J/cm^2) altered the expression of the proteins involved in the regulation of apoptosis, stress response and cell cycle. This change resulted in a significant increase in cell photo-tolerance.

Key words: Photodynamic therapy; Heat shock protein; Cell cycle; Apoptosis; Endometrial carcinoma

1. Introduction

Photodynamic therapy (PDT) is a relatively young cancer treatment based on the application (topic or systemic) of a photosensitizing agent that accumulates in target tissues [1,2]. Upon irradiation with tissue-penetrating light, the activated photosensitizer produces reactive oxygen species that lead to cell death. The mechanisms that control cell death vary according to cell type and the type of photosensitizer [3]. Advances in PDT will probably depend on the discovery of new chromophores and the characterization of those already being used. The natural non-toxic chromatophore hypericin [4] is attracting renewed attention because of its potential as a photosensitizing anti-cancer drug. Indeed, neoplastic cells in culture respond to hypericin in a dose-dependent fashion: high doses of light and high concentrations of photosensitizer cause apoptosis or cell necrosis [5], whereas low levels of activation induce only a 'stress response' [6] that involves the synthesis of proteins known as stress-induced or heat shock proteins (HSP) [7]. The main function of HSP is to afford additional protection to the cell as the need arises. Induction of HSP appears to coincide with acquisition of tolerance to high levels of stress that would otherwise kill the cell [8,9].

Because PDT induces cell damage via oxidative stress, we investigated, in HEC1-B cells, whether sub-lethal photosensitization confers resistance against further photo-induced damage, reduces apoptosis and preserves cell proliferation.

2. Materials and methods

2.1. Cells, chemicals, laser source

HEC1-B human endometrial carcinoma cells (American Type Cell Culture Collection, ATCC, MD, USA) were cultured as previously described [10]. Their doubling time is >36 h [10]. 1 mg/ml hypericin in dimethylsulfoxide was used as stock solution. Experiments were routinely performed by diluting this stock to 0.15 μM.

A coherent light at 599 nm (corresponding to a major absorption peak of hypericin) was obtained from a laser dye (Coherent model CR-599-01, Palo Alto, CA, USA) pumped by a continuous wave argon laser (model CR-18Sg). Power density was measured with a Ophyr, model DGX 10 power meter.

2.2. Cells photosensitization

Before irradiation, cells (1.5×10^6 in 3 ml of medium) were incubated in 60-mm Petri dishes for 16 h with 0.15 μM hypericin. Plates were washed twice with hypericin-free medium and positioned under the laser beam at the appropriate distance. The temperature of plates during irradiation (3–15 min) was controlled by their partial immersion in a water bath heated at 35°C. Cells were irradiated either with single light doses (2.0, 3.5, 5.0 and 10.0 J/cm^2) or two successive doses (i.e. 2.0 and 5.0 J/cm^2) spaced by 3 or 20 h. After exposure(s), cells (triplicates) were incubated in the dark at 37°C for 7 h before final analysis.

2.3. Hypericin loading, sub-cellular distribution and release

Loading. About 1.5×10^6 cells were incubated for 16 h with 0.15 μM hypericin. Hypericin was assayed fluorimetrically as described by Paba et al. [9]. To study the sub-cellular distribution of hypericin, HEC1-B cells were sub-fractionated essentially as described by Hovius et al. [11] and Dignam et al. [12]. Proteins c-Myc and actin served as markers for the nuclear and cytosolic fractions, respectively; proteins p-Bip and VDAC [13,14] served as markers for endoplasmic reticulum (ER) and mitochondrial fractions, respectively. Hypericin fluorescence in individual fractions was normalized with respect to protein content.

2.4. Fluorescence-activated cell sorter (FACS) analysis

Experiments with asynchronous cultures were done on a FACScan (Becton-Dickinson) with 3×10^6 HEC1-B. Cells were repeatedly washed with phosphate saline buffer (PBS) pH 7.4 and fixed in 75% ethanol for 16 h at −20°C. After further washing with PBS containing RNase (0.1 mg/ml, final), cells were stained with 20 mg/ml propidium iodide solution for 30 min at room temperature. Data were analyzed with dedicated software (Cell-Fit).

2.5. Protein extraction, electrophoresis and Western blot

Protein extraction from cells, electrophoresis and protein transfer technique have been described elsewhere [15]. Western immunoblottings and chemiluminescence detection of poly(ADP-ribose)polymer-

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ase (PARP), Bcl-2, Bax, p21, p53, HSP70, c-Myc, p-Bip, VDAC and β-actin were performed as previously described [9,10,15]. The expression of β-actin was also used to check protein loading.

3. Results and discussion

3.1. Hypericin photoactivation can induce apoptosis or necrosis in HEC1-B cells depending on the strength of activation (light dose and hypericin concentration)

HEC1-B cells were cultured for 16 h with 0.15 μM hypericin, washed and exposed to light doses of 0, 2.0, 3.5, 5.0 and 10.0 J/cm^2. Seven hours later, the integrity of PARP, a well-recognized early marker of apoptosis, was analyzed in cell extracts by Western blot. Light doses between 2.0 and 5.0 J/cm^2 caused a progressive decrease in the amount of intact protein (115 kDa) and a parallel accumulation of the 85-kDa remnant (Fig. 1A). Higher doses of light caused a proportional increase in the number of cells committed to apoptosis. In fact, 2.0 J/cm^2 did not damage the cell, as demonstrated by unchanged cell morphology as well as by PARP integrity, whereas at 5.0 J/cm^2, the significant amount of PARP cleavage fragment and a large number of apoptotic bodies at microscope inspection (data not shown) indicate an impending apoptotic process. Between 6 and 10.0 J/cm^2, PARP fragments were no longer detected, but there were morphological signs of severely damaged cells. It is feasible that the massive photo-induced injury causes the extensive and rapid denaturation of cell enzymes thereby leading to necrosis. Similar findings have been described in other cell systems [5].

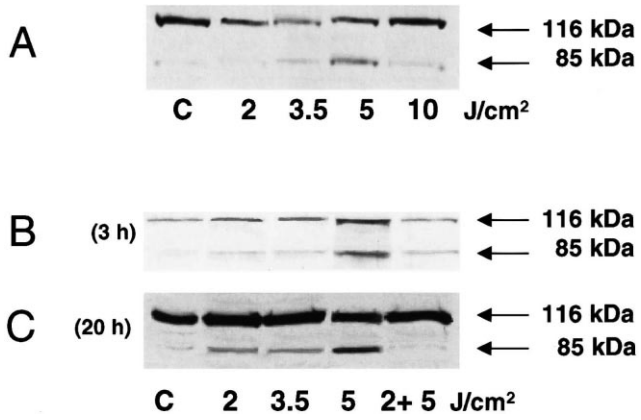

Fig. 1. A–C: PARP cleavage in protein extracts of HEC1-B cells after photoactivation of cells incubated for 16 h with 0.15 μM hypericin at 599 nm at different light doses (as indicated). When two irradiations were used (B and C, lanes 5), the intervals between exposures were 3 h (B) and 20 h (C).

3.2. Effect of irradiation at 5.0 J/cm^2 on hypericin-loaded cells previously exposed to low light doses (2.0 J/cm^2)

We next evaluated the effect on hypericin-loaded cells of pre-sensitization with a light dose of 2.0 J/cm^2 (apparently innocuous) followed by 5.0 J/cm^2 (apoptosis-inducing dose) shortly after (3 h) or much later (20 h) when the effects of pre-sensitization had probably subsided. PARP cleavage was modest after 3 h (Fig. 1B). At an interval of 20 h PARP cleavage was absent (Fig. 1C, lane 5). To address the possibility that absence of cleavage was due to diffusion of hyper-

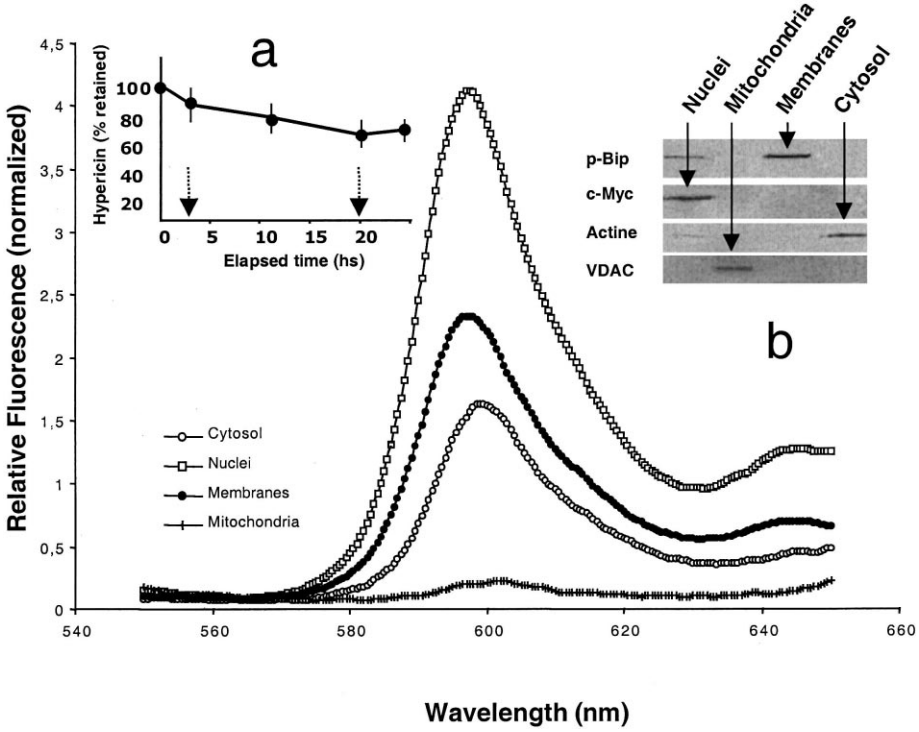

Fig. 2. Normalized fluorescence emission spectra (550–650 nm) of hypericin from individual sub-cellular compartments of hypericin-loaded HEC1-B cells. a: Relative fluorimetric estimate of cell-trapped drug at indicated times. The arrows indicate the residual hypericin fluorescence at 3 and 20 h. Hypericin was measured fluorimetrically in three experiments and expressed as percent (±S.D.) with respect to hypericin fluorescence obtained at time 0. b: Sub-cellular fractionation was validated by Western blot using protein markers specific for each fraction: actin (cytosol), c-Myc (nucleus), VDAC (mitochondria) and p-Bip (membranes).

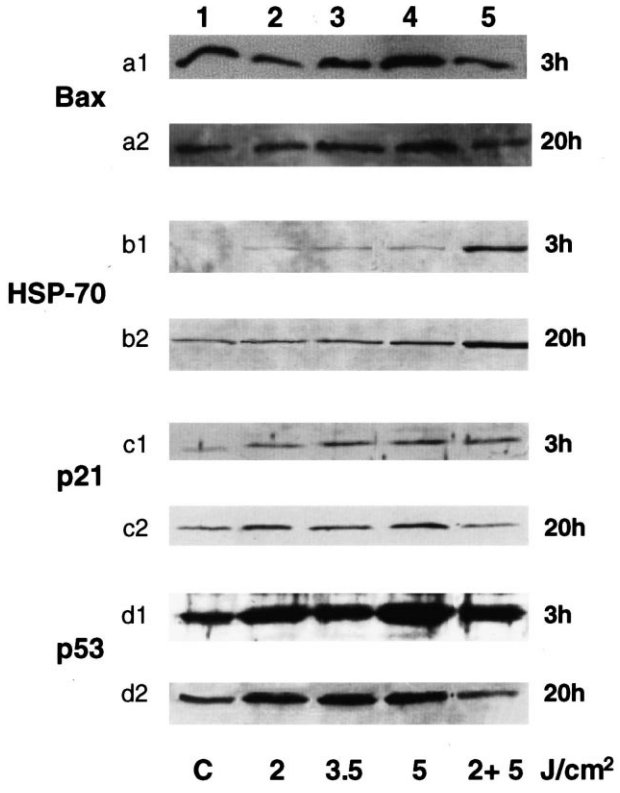

Fig. 3. a and b: Effects of hypericin photoactivation on the expression of Bax and HSP70. Lanes 1–4: Bax and HSP70 expression in cells exposed to light fluences of 0, 2, 3.5 and 5 J/cm^2. Lanes 5: Bax and HSP70 expression in cells sensitized with a low light dose (2 J/cm^2) and successively challenged with a more intense light fluence (5 J/cm^2) after 3 h (a1 and b1) or 20 h (a2 and b2). c and d: Effects of hypericin photoactivation on the expression of p21 (c1 and c2) and p53 (d1 and d2). Lanes 1–4: protein expression in cells exposed to light fluences of 0, 2, 3.5 and 5 J/cm^2. Lane 5: expression of p21 and p53 protein cells sensitized with a low light dose (2 J/cm^2) and successively challenged with a more intense light fluence (5 J/cm^2) after 3 h (c1 and d1) or 20 h (c2 and d2).

icin in the medium between irradiations, we studied hypericin uptake, release and sub-cellular distribution in HEC1-B cells.

3.3. Hypericin uptake, release and sub-cellular distribution in HEC1-B cells

As shown in Fig. 2 (insert a) hypericin uptake remained quite steady from 3 to 20 h. In fact, more than 75% of the hypericin present at 3 h was available and susceptible to sensitization at 20 h. To determine the sub-cellular distribution of hypericin, we performed cellular sub-fractionation of hypericin-loaded HEC1-B cells, by using ultracentrifuge-based methods [11,12]. We finally verified this procedure by checking for the specific marker proteins c-Myc (nuclear), p-Bip (ER, [13]), actin (cytosol) and VDAC (mitochondria, [14]) within each sub-fraction (Fig. 2, insert b). The amount of hypericin in sub-cellular fractions was estimated fluorimetrically (emission spectra) and expressed after normalization (Fig. 2). Hypericin tended to accumulate in the nuclear fraction (Fig. 2b). This finding strongly suggests that hypericin sensitization and decay cause locally DNA damage. Thus, we investigated possible alterations in the expression of some crucial proteins which are normally related to cellular damage.

3.4. Altered expression of specific proteins

The data reported in Fig. 1A suggested that exposure of hypericin-preloaded cells to increasing light doses involves a switch from apoptosis to necrosis – an effect already demonstrated in HeLa and U937 cells [5,9]. Therefore, we studied the expression of proteins involved in apoptosis, stress, and the cell cycle (Bax, Bcl-XL, HSP70, p21 and p53) after exposure to single and double irradiation.

3.5. The Bax and Bcl-XL proteins

Single exposure to 3.5 and 5.0 J/cm^2 irradiation caused only moderate changes in Bax expression (1.2–1.4-fold increases over controls) (Fig. 3, a1 and a2, lanes 1–4); there were no changes when the initial irradiation (2.0 J/cm^2) was followed by irradiation at 5.0 J/cm^2 (lane 5). The expression of Bcl-XL, which controls resistance to apoptosis in HEC1-B cells [10], was unchanged (not shown). These findings, indeed, are in agreement with data on PARP cleavage previously shown. Lastly, while data suggest a Bax-driven apoptosis from 3.5 up to 5.0 J/cm^2 (single exposures), this is not the case when the cells are irradiated twice, and particularly with a long interval (20 h) between the two irradiations.

3.6. HSP70 protein

There is strong evidence that HSP induction coincides with acquisition of tolerance to stress which otherwise may kill the cell [8]. For example, heat-shocked cells are more resistant to environmental stress and death [7,16]. Similarly, apoptosis induced by various stresses is inhibited in heat-shocked cells suggesting that HSP play a role in resistance mechanisms [17].

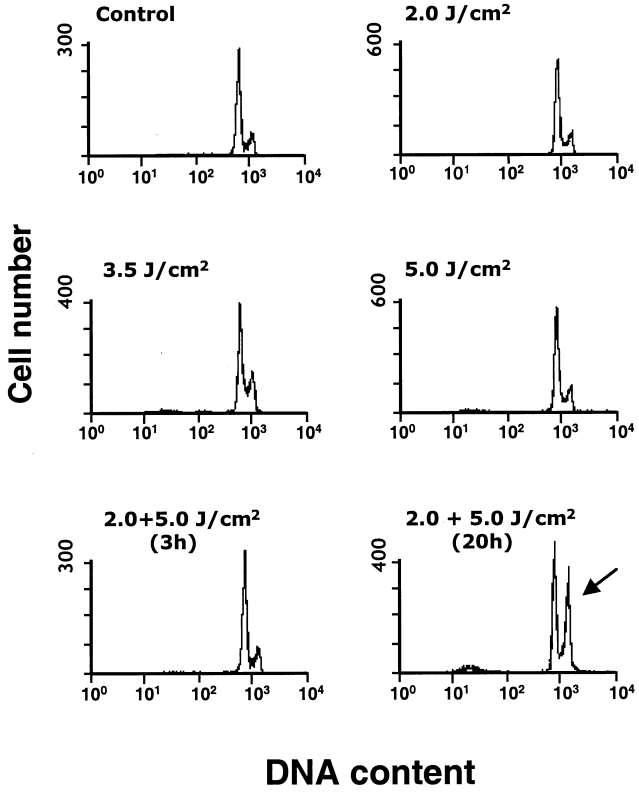

Fig. 4. Flow cytometric profiles of HEC1-B cells at various fluences. The two lower panels differ in the interval between the first and the second exposure to light (i.e. 3 or 20 h). The arrow remarks the cell accumulation in the G2/M observed at the indicated conditions.

Hypericin photoactivation stimulated HSP70 synthesis in a dose-dependent fashion (Fig. 3, b1 and b2). Previous photoactivation with 2 J/cm^2 greatly enhanced HSP70 expression (Fig. 3, b1 and b2, lane 5). Paba and colleagues [9] obtained similar results in U937 cells using the same sensitizer but at much higher doses. HSP70 did not increase further at an inter-exposure interval of 20 h. We analyzed protein expression about 7 h after the second exposure to light, which is sufficient time for de novo synthesis of HSP70 proteins.

3.7. The p53 and p21 proteins: cell cycle

Sub-fractionation of HEC1-B cells and dye assay by fluorescence showed that hypericin tends to accumulate in the nucleus. Indeed, hypericin photosensitization damages cells through a type II photochemical pathway that involves the generation of singlet oxygen [18]. This species is highly reactive and, being formed in the nucleus, it can damage the HEC1-B DNA, which is a preferential bio-substrate.

Protein p53 senses DNA damage at several stages of the cell cycle and determines whether the cell cycle should stop for DNA repair or, if this is not possible, it triggers apoptosis [19]. Indeed, protein p53 is a sequence-specific transcription factor that coordinately activates the transcription of downstream effector genes. Protein p21^{CIP1}, a well-studied cell cycle inhibitor, is a relevant downstream effector gene [20]. It is not inconceivable that photoactivation-induced damage might result in changes in the expression of both proteins p53 and p21.

Analysis of cell extracts by Western blot using specific antibodies against p21 and p53 proteins demonstrated that at doses of light between 2 and 5 J/cm^2, the expression of both proteins increased with respect to the control (Fig. 3, c1 and c2, d1 and d2, lanes 1–4), which indicates activation of a repair/defense cell response. However, when cells were exposed to light twice, p21 and p53 expression was practically unchanged at 3 h (Fig. 3, c1 and d1, line 5) and clearly reduced at 20 h (Fig. 3, c2 and d2, line 5). The decreased expression of both proteins at the longer time cannot be easily explained. Possibly, at the second exposure (20 h), while HPS levels are elevated, p53 and p21 synthesis is no longer needed because the cell cycle is slowing down and cellular rescue mechanisms were fully activated by the first photosensitization. We tested this hypothesis by analyzing the distribution of cell populations after single and double irradiation. It appears that irradiation causes HEC1-B cells to accumulate in G2/M phase. The mildly sensitized cells, upon further activation, go toward cell cycle arrest. This block becomes evident 20 h after the second exposure (Fig. 4, lowest right panel). Indeed, although previous findings indicated that p53 causes cell arrest in G1 phase [21], more recent data have shown that p53 can also induce arrest in G2/M phase [22].

Our findings sustain the hypothesis that hypericin sensitization induces damage that causes apoptosis or cell necrosis according to the energy adsorbed. Finally, we demonstrate that mild pre-sensitization endows cells with an unexpected high degree of photo-tolerance, enhances HSP70 synthesis, sequentially promotes the expression of specific apoptosis-related proteins and causes cell cycle arrest.

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