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A role for the transient increase of cytoplasmic free calcium in cell rescue after photodynamic treatment

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Chinese hamster ovary (CHO) cells and T24 human bladder transitional carcinoma cells were treated with the photosensitizers aluminum phthalocyanine (AlPc) and hematoporphyrin derivative (HPD), respectively. Exposure of both sensitized cell lines to red light caused an immediate increase of cytoplasmic free calcium, $[Ca^{2+}]_i$, reaching a peak within 5–15 min after exposure and then returning to basal level (~ 200 nM). The level of the peak $[Ca^{2+}]_i$ depended on the light fluence, reaching a maximum of 800–1000 nM at light doses that kill about 90% of the cells. Loading the cells with the intracellular calcium chelators quin2 or BAPTA prior to light exposure enhanced cell killing. This indicates that increased $[Ca^{2+}]_i$ after photodynamic therapy (PDT) contributed to survivability of the treated cells by triggering a cellular rescue response. The results of experiments with calcium-free buffer and calcium chelators indicate that both in CHO cells treated with AlPc and with HPD-PDT of T24 cells extracellular Ca^{2+} influx is mainly responsible for elevated $[Ca^{2+}]_i$. PDT is unique in triggering a cell rescue process via elevated $[Ca^{2+}]_i$. Other cytotoxic agents, e.g., H_2O_2, produce sustained increase of $[Ca^{2+}]_i$ that is involved in the pathological processes leading to cell death.

Introduction

The use of the photosensitizer Photofrin^R and red light for the treatment of various forms of cancer is termed photodynamic therapy (PDT) and is now in phase III clinical trials [1]. Second generation sensitizers for PDT are being studied in order to overcome the shortcomings of Photofrin [2]. Phthalocyanines are efficient photosensitizers in experimental PDT and are about to enter phase I clinical trials [3,4]. The molecular and cellular mechanisms by which these sensitizers cause cytotoxicity are largely unknown. To improve the efficacy of PDT it is of the utmost importance to understand its biochemical background. Cytotoxicity depends, among other factors, on the presence of oxygen [5–7]. Oxygen is thought to mediate damage in critical targets via singlet oxygen and probably other reactive oxygen species [8,9].

Cytotoxicity induced by various xenobiotics appears to involve elevated $[Ca^{2+}]_i$ in many cases [10,11]. A causal relationship is usually deduced from cytoprotection by calcium chelators that can permeate the cell membrane, e.g., quin2/AM [12]. Sustained increase of $[Ca^{2+}]_i$ could lead to cell death by various pathways: perturbation of cytoskeletal organization, activation of degradative enzymes (phospholipases, proteases and endonucleases) and impaired mitochondrial function [11]. Recently, a transient increase of $[Ca^{2+}]_i$ was observed following AlPc-PDT [13]. However, a causal relationship with cell killing was not established in this case.

The purpose of this study was to establish a role for elevated $[Ca^{2+}]_i$ in PDT-induced cytotoxicity and to characterize the $[Ca^{2+}]_i$ changes in 2 cell lines using HPD and AlPc. Unexpectedly, $[Ca^{2+}]_i$ chelators enhanced cell killing in both cases, indicating that the $[Ca^{2+}]_i$ transient after PDT has a protective role. This

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Abbreviations: AlPc, aluminum phthalocyanine chloride; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester; $[Ca^{2+}]_i$, cytoplasmic free calcium; DMSO, dimethylsulfoxide; DPBS, Dulbecco's phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HPD, hematoporphyrin derivative; PDT, photodynamic therapy.

is the first report to demonstrate such a role for a transient elevation of $[Ca^{2+}]_i$.

Materials and Methods

Chemicals. Aluminum phthalocyanine chloride (AlPc) (MW 574.97) was obtained from Eastman Kodak, Rochester, NY, and was stored as a stock solution of 1 mM in dimethylformamide at room temperature. HPD was from Paisley Biochemicals, Paisley, UK and was stored as a stock solution (5 mg/ml) in saline at −20°C. Fura-2/AM, verapamil, quin2/AM and the ionophore A23187 were purchased from Sigma, St Louis, MO, USA. BAPTA/AM was from Molecular Probes, Eugene, OR, USA. The calcium reagents were stored as stock solutions of 2 mM in DMSO at −20°C. Radioactive $^{45}CaCl_2$ (70.3 MBq/ml) was obtained from Amersham, Amersham, UK. All other chemicals were of analytical grade.

Cell culture. Chinese hamster ovary cells, clone K1, ATCC number CCL61 and human bladder transitional carcinoma cells clone T24, ATCC number HTB4, were grown attached to plastic petri dishes in HAM's F10 medium, supplemented with 15% newborn calf serum, in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell survival. Chinese hamster cells were plated in 5 cm dishes in appropriate numbers (200–20000) to obtain 100–200 colonies after treatment. After 4 h AlPc was added and incubation continued for 2 h. This time of incubation insured maximal photosensitization. The medium was then replaced with 2 ml DPBS and the cells were exposed to light. Growth medium was added back and incubation continued for 7 days. Colonies were then stained and counted. T24 cells were incubated with 10 μg/ml HPD in DPBS for 1 h at 37°C prior to light exposure. After the treatment the cells were trypsinized, plated (400 cells/dish) and incubated 10 days for colony formation [14]. Triplicate plates were used for each datum point. Standard errors were less then 10%.

Light exposure. Cells loaded with AlPc were illuminated with a slide projector equipped with a 150 W quartz halogen light bulb. The light was filtered by a cut-off filter ($\lambda > 605$ nm). The incident fluence rate, measured in this wavelength region, was 120 W/m^2. HPD-treated cells were exposed to red light (20 W/m^2) from a 500 W halogen lamp filtered by a cut-off filter ($\lambda > 590$ nm) and a 1-cm thick circulating water layer to avoid hyperthermia effects.

$[Ca^{2+}]_i$ measurement. Assay of $[Ca^{2+}]_i$ was as previously described [15], with some modifications. Cells were loaded with fura-2/AM at room temperature by addition into the growth medium at 2 μM for 30 min before measurement of fluorescence. Prior to the assay the cells (10^6 cells/5 cm dish) were rinsed three times with DPBS and suspended in 3 ml DPBS by scraping with a rubber policeman. Fluorescence of the cell suspension was measured in an Aminco SPF 500 spectrofluorometer at two excitation wavelengths, 340 nm and 385 nm, with emission measured at 505 nm. The ratio F_{340}/F_{385} was used to calculate $[Ca^{2+}]_i$ as described by Grynkiewicz et al. [16]. This ratio was corrected for the absorbance of the sensitizers in this region.

In control experiments it was shown that both the fluorescence of fura-2 and of fura-2/Ca was not influenced by photodynamic effects. The calcium chelators had no effect on the photodynamic potention of the sensitizers, as measured by the oxygen consumption in the presence of either histidine or tryptophan.

Ca-uptake. After photodynamic treatment the buffer was replaced by culture medium containing 1 μCi ^{45}Ca/ml. Uptake was terminated by washing the cells five times with ice-cold buffer, containing 140 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 5 mM Hepes and 10 mM $LaCl_3$ (pH 7.4) [17]. Thereafter the cells were scraped off in DPBS. An aliquot of the suspension was diluted with Packard Scintillator 299™ and the radioactivity was determined with a Packard Tri-Carb 4000 scintillation counter.

Protein. Protein was determined according to Lowry et al. [18].

Results

Fig. 1 shows the changes in $[Ca^{2+}]_i$ as a function of time after AlPc-PDT in Chinese hamster cells. An immediate increase in $[Ca^{2+}]_i$ was observed reaching a peak at about 4 min. The magnitude of increase was light fluence dependent. Maximal response occurred

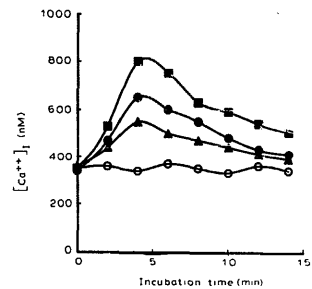

Fig. 1. Changes in $[Ca^{2+}]_i$ in CHO cells incubated for 2 h with 1 μM AlPc. Control, unilluminated cells, ○; cells illuminated for 5 s, ▲; 15 s, ● and 45 s, ■.

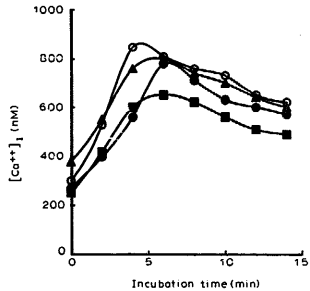

Fig. 2. Changes in $[Ca^{2+}]_i$ in CHO cells incubated for 2 h with 1 μM AlPc and then exposed to 45 s red light. Control, no additional treatments, ○; 0.5 mM EGTA added to growth medium after illumination, ●; 20 μM verapamil added for 30 min prior to and after illumination, ■; 5 mM NaF during illumination only, ▲.

after 45 s of illumination (Fig. 1). Higher light fluences caused no further increase of $[Ca^{2+}]_i$. The time at which $[Ca^{2+}]_i$ levels returned to basal values increased from about 15 min after 5 s of light exposure to about 2 h after 45 s of illumination (not shown). To determine if influx of external Ca^{2+} was the source of elevated $[Ca^{2+}]_i$ after AlPc-PDT, cells were incubated after PDT with 0.5 mM EGTA to chelate external calcium. Under these conditions EGTA reduces external calcium to 80 nM [17]. Also incubation with 20 μM verapamil was done, to block calcium channels. At this concentration verapamil protects against Ca^{2+}-related cell death [19]. Both treatments caused some reduction of $[Ca^{2+}]_i$ but did not eliminate the response (Fig. 2). No rise in $[Ca^{2+}]_i$ could be observed however, when after illumination the cells were covered with DPBS containing 1 mM EGTA ($[Ca^{2+}]_i < 1$ nM). Fig. 2 also shows that 5 mM NaF, which protects against cytotoxicity induced by AlPc-PDT [13], did not prevent the transient increase of $[Ca^{2+}]_i$. From Fig. 3 it is clear that the uptake rate of calcium from the medium was decreased after AlPc-PDT.

To test whether elevated $[Ca^{2+}]_i$ is involved in phototoxicity the cells were loaded with either one of the Ca^{2+} chelators quin2 or BAPTA prior to light exposure. Loading is done using the ester form (AM) of the chelators, which can freely permeate the plasma membrane. The ester is rapidly hydrolyzed in the cytoplasm to the free acid that chelates calcium. The hydrolyzed chelators are trapped inside the cell. The results show that neither quin2 nor BAPTA protected against cell killing. On the contrary, both enhanced it considerably (Fig. 4), changing the LD_{90} from an illumination time of 45 s to about 25 s. Reduction of external calcium by

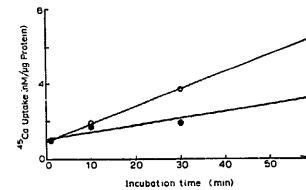

Fig. 3. Uptake of $^{45}Ca^{2+}$ by CHO cells before (○) and after 45 s of AlPc-PDT (●) as a function of time.

EGTA (Fig. 4) or blocking calcium channels with verapamil (not shown) did not affect cell survival. Further correlation between the $[Ca^{2+}]_i$ transient and cell survival was obtained by analyzing the kinetics of the disappearance of the sensitizing effect of BAPTA/AM (Fig. 5). In this experiment BAPTA/AM was added at various times after illumination. The results show that cells regained their normal response to AlPc-PDT at a fast rate, comparable to the changes in $[Ca^{2+}]_i$. Since it may take up to 10 min for BAPTA to reach intracellular levels sufficient to chelate all $[Ca^{2+}]_i$ [20], the curve in Fig.5 should be displaced 10 min to the right to reflect this fact.

To test whether the protective effect of calcium was a peculiarity for AlPc-photosensitized CHO cells, similar experiments were performed with HPD-sensitized T24 cells. This system mimics the clinical situation more closely as T24 is a line of bladder carcinoma, which is being treated by PDT in the clinic, and HPD

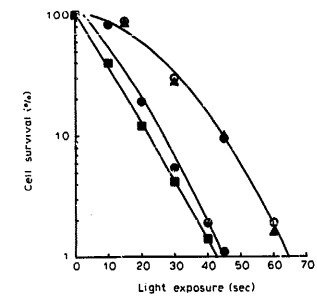

Fig. 4. Survival of CHO cells incubated for 2 h with 0.5 μM AlPc and then exposed to red light. Control, no additional treatments, ○; 10 μM quin2/AM for 1 h prior to illumination, ●; 10 μM BAPTA/AM for 1 h prior to illumination, ■; 0.5 mM EGTA for 2 h during and after illumination, ▲.

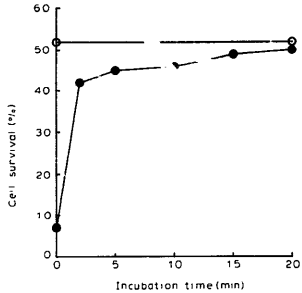

Fig. 5. Survival of CHO cells incubated for 2 h with 0.5 μM AlPc and then exposed for 30 s to red light (●). BAPTA/AM (10 μM) was added for 1 h before light exposure (zero time) or at various times after illumination, as indicated. The horizontal line (○) indicates survival of cells irradiated, but not treated with BAPTA/AM.

is the only clinically approved sensitizer. A transient increase in $[Ca^{2+}]_i$ immediately after illumination is shown in Fig. 6. $[Ca^{2+}]_i$ reached a maximum after 10 min light exposure and decreased to control level after prolonged illumination times. The initial concentration of less than 200 nM increased to about 500 nM. The changes in $[Ca^{2+}]_i$ as a function of time after light exposure are shown in Fig. 7. A dose-dependent increase was observed, reaching a maximum at 15 min post-PDT. After 2 h incubation in growth medium the $[Ca^{2+}]_i$ was reduced to control levels of less than 200

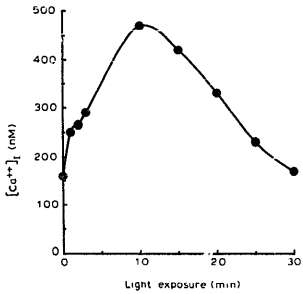

Fig. 6. Dose response of $[Ca^{2+}]_i$ changes after HPD-PDT in T24 cells. Cells were incubated with HPD and exposed to light as described in Materials and Methods. Immediately after the indicated light exposures the level of $[Ca^{2+}]_i$ was measured.

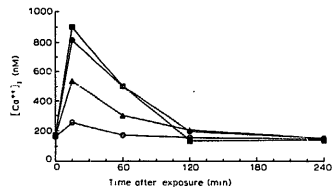

Fig. 7. Kinetics of $[Ca^{2+}]_i$ changes after HPD-PDT in T24 cells. Cells were incubated with HPD and then exposed to various light fluences. The level of $[Ca^{2+}]_i$ was measured at various times after light exposure. Dark control, ○; 5 min light, ▲; 10 min light, ●; 15 min light, ■.

nM. In Fig. 8 the involvement of increased $[Ca^{2+}]_i$ levels in cell survival after HPD-PDT is depicted. Loading the cells with quin2/AM prior to light exposure enhanced cell killing. The LD_{90} dropped from an illumination time of 13 min to 9 min. A comparable enhancement of cytotoxicity was measured when the cells were pre-incubated with 20 μM of the calcium channel blocker verapamil or when 0.5 mM EGTA was added for a period of 6 h subsequent to light exposure (Fig. 8). The calcium ionophore A23187 at 5 μM protected T24 cell against cytotoxicity of HPD-PDT.

Fig. 9 shows that in CHO cells treated with H_2O_2 the kinetics of $[Ca^{2+}]_i$ changes are quite different than those observed after PDT. $[Ca^{2+}]_i$ was increased in a

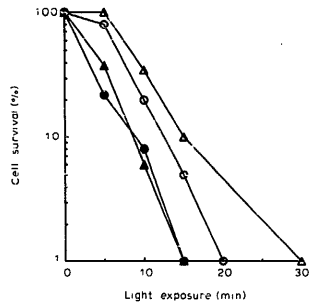

Fig. 8. Survival of T24 cells treated with HPD-PDT. Drug and light treatments were as described in Materials and Methods. Control, no additional treatments, ○; cells incubated with 10 μM quin2/AM for 1 h prior to light exposure, ●; cells incubated with 0.5 mM EGTA for 6 h during and after light exposure, ▲; cells incubated for 1 h with 5 μM A23187 prior to light exposure, △.

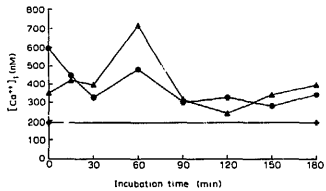

Fig. 9 Changes in $[Ca^{2+}]_i$ in CHO cells treated with H_2O_2. Cells were incubated with 750 μM (●) or 500 μM H_2O_2 (▲) for 1 h. $([Ca^{2+}]_i)$ was measured at various times after treatment, as described in Materials and Methods.

dose-dependent manner and sustained for a at least 3 h. Addition of BAPTA/AM protected the cells from toxicity of H_2O_2 (Fig. 10).

Discussion

$[Ca^{2+}]_i$ is a second messenger that regulates a large number of physiological processes in animal cells [21]. Recently, it became clear that elevated levels of $[Ca^{2+}]_i$ can play a role in a variety of pathological and toxicological processes [11]. Most of this work was done with isolated hepatocytes and protection against cytotoxicity was assayed with short term tests, e.g., lactate dehydrogenase release. Questions were therefore raised concerning the hypothesis that $[Ca^{2+}]_i$ increase may be a common step in the development of cytotoxicity [10]. A convincing case for the role of $[Ca^{2+}]_i$ in lethal cell injury using clonogenic cell survival assay was made in the case of H_2O_2-treated CHO cells (Fig. 10), in which

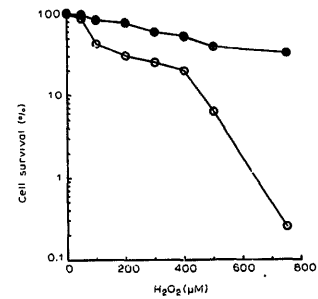

Fig. 10. Survival of CHO cells treated with H_2O_2. Cells were incubated with the indicated concentration of H_2O_2 in DPBS for 1 h at 37°C. Prior to treatment the cells were incubated for 1 h in the absence (○) or in the presence (●) of 10 μM BAPTA/AM.

BAPTA/AM protected against cytotoxicity. This is in agreement with a previous report [12], using quin2/AM and cell growth as an assay, and is consistent with the sustained increase of $[Ca^{2+}]_i$ (Fig. 9).

Elevation of $[Ca^{2+}]_i$ after PDT has been described before by Specht and Rodgers [22]. The present experiments show a marked transient elevation of $[Ca^{2+}]_i$ following PDT in two cell lines and using two sensitizers (Figs. 1 and 7). In both cases loading the cells with calcium chelators prior to PDT enhanced cell killing (Figs. 4 and 8). The chelators are only present during illumination and a short period thereafter, until they have leaked out. The effect on survival can only have been effected during the period that they were present. This is the first report indicating that the $[Ca^{2+}]_i$ transient elevation serves as a signal in triggering a cellular response that diminishes PDT-induced damage. The observation that the sensitizing effect of BAPTA/AM in CHO cells treated with AlPc-PDT disappeared within a short time after illumination (Fig. 5) strongly supports a role for the $[Ca^{2+}]_i$ transient in maintaining cell survival. It is of interest that the effect of $[Ca^{2+}]_i$-chelators is to remove the shoulder of the survival curves. Thus, the cellular process triggered by $[Ca^{2+}]_i$ enables the cells to accumulate sublethal damage. Since the cells are able to repair HPD-PDT as well as AlPc-PDT sublethal damage [23,24] it is tempting to speculate that the $[Ca^{2+}]_i$ transient is a signal for the induction of such a repair process.

The source of the calcium increase after AlPc-PDT in CHO cells appears to be the external medium, as is clearly indicated by the lack of a $[Ca^{2+}]_i$ rise when a Ca-free buffer was used. This fact, together with the observation that the calcium influx was decreased after PDT (Fig. 3), indicates that a lowered calcium pump activity is involved in the transiently increased $[Ca^{2+}]_i$. CHO cells apparently have the ability to specifically or actively take up calcium, because in the presence of very low calcium concentrations, as in the EGTA-containing culture medium, the increase in $[Ca^{2+}]_i$ by AlPc-PDT is only moderately lower than in a medium without EGTA. Apparently the increase in this case is still sufficient to trigger the protective cellular response.

Also in HPD-PDT treated T24 cells $[Ca^{2+}]_i$ elevation was due to influx of extracellular calcium. In contrast with AlPc-PDT treated CHO cells, EGTA in the culture medium prevented the transient elevation of $[Ca^{2+}]_i$ and consequently cell killing was enhanced.

Another difference between AlPc-PDT and HPD-PDT was the shape of the dose–response curves for elevation of $[Ca^{2+}]_i$. With AlPc-PDT and CHO cells $[Ca^{2+}]_i$ reached its maximum value after 45 s of illumination without a significant lower value after higher doses, while with HPD-PDT of T24 cells a bell-shaped curve was observed with a maximum at 10 min illumi-

nation (Fig. 6). The reduction of the response in the latter case after longer exposures is unlikely to be due to inhibition of influx of Ca^{2+}. It probably reflects the kinetics of the $[Ca^{2+}]_i$ changes, which peak at 10–15 min post PDT (Fig. 7). The decay of $[Ca^{2+}]_i$ levels are most likely due to the action of the Ca^{2+} pump that maintains Ca^{2+} homeostasis in the cell.

The protection of T24 cells incubated with the calcium ionophore prior to HPD-PDT (Fig. 8) is consistent with a role for elevated $[Ca^{2+}]_i$ in survivability of PDT-treated cells. The most plausible explanation is induction of the yet unknown cellular process that is responsible for enhanced survival, by the increase of $[Ca^{2+}]_i$ produced by A23187. The increase of $[Ca^{2+}]_i$ prior to PDT is apparently more favorable for cell survival than the later occurring rise caused by PDT itself.

It is noteworthy that cytotoxicity of AlPc-PDT and the induction of a $[Ca^{2+}]_i$ transient can be dissociated. Thus, F^- can protect against CHO cell killing by AlPc-PDT [13] but caused only a small reduction of the $[Ca^{2+}]_i$ response (Fig. 2). It appears, therefore, that while fluoride protects critical target(s), presumably the Na^+/K^+-ATPase (to be published elsewhere), it does not protect against production of damage that triggers the $[Ca^{2+}]_i$ increase. The increase of the basal level of $[Ca^{2+}]_i$ by F^- only, was a consistent finding and is also observed in other cellular systems [25,26].

In conclusion, a transient elevation of $[Ca^{2+}]_i$ is observed both in AlPc-PDT treated CHO cells and in HPD-PDT treated T24 cells. The $[Ca^{2+}]_i$ transient appears to induce a process that enhances the ability of the cells to survive a PDT treatment. PDT is unique in this respect among the cytotoxins that have been studied so far. Further experiments will be necessary to elucidate the biochemical mechanisms involved in the protecting effect of increased cytoplasmic Ca^{2+} concentrations.

Acknowledgements

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