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MINI REVIEW

Mitochondria are targets of photodynamic therapy

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Abstract Photodynamic Therapy (PDT) is an evolving cancer treatment that depends on three known and variable components: photosensitizer, light and oxygen. Optimization of these variables yields reactive oxygen species, mainly singlet oxygen, that damage cellular components leading to cytotoxicity. Our research has demonstrated that porphyrin sensitizers, in particular, significantly inhibit the inner mitochondrial membrane enzymes cytochrome c oxidase and F_0F_1 ATP synthase. These results were obtained from an *in vivo-in vitro* experimental protocol that exposes sensitizers to metabolic and pharmacokinetic events. The resulting inhibition of oxidative phosphorylation was expected to reduce ATP levels, which were quantitated in cells and were confirmed by ^{31}P-NMR spectroscopy of tumors *in situ* in animals treated with PDT.

Based on these findings, and more recent investigations of apoptosis, there is little doubt that mitochondria are critical targets in the actions of PDT.

Keywords Photodynamic therapy · Mitochondrial targets · Enzyme inhibition · ATP levels · Tumor cytotoxicity

Results and discussion

Photodynamic Therapy (PDT) is an evolving cancer treatment regimen that is comprised of three components: photosensitizing agent; visible light; and presence of oxygen. Much of the research has been directed towards optimization of these components, seeking to establish parameters of maximum efficacy for application in clinical settings. Several reviews of PDT have appeared, wherein descriptions of the effects of purified hematoporphyrin preparations, novel synthetic sensitizers, and precursors or pro-drugs (such as aminolevulinic acid or ALA) have been investigated. Attention to physical parameters of light, such as wavelength, fluence rates and continuous versus intermittent regimens have been explored. Since it is well accepted that the photochemistry of light activation of these sensitizers leads to production of reactive oxygen species (ROS), particularly singlet oxygen and/or hydroxyl radicals and peroxides, efforts have been directed to document and perhaps modify the presence of hypoxia in tumor regions that might preclude the production of ROS and their cytotoxic actions that form the basis of reduced neoplastic growth. However, when we first became interested in PDT, there was little in the literature that addressed mechanisms leading to tumor inhibition.

In 1982, a report by Berns et al. (1982) examined cellular effects of hematoporphyrin derivative (HpD) in several established cell lines. Investigation of these non-cancerous cells *in vitro* by fluorescence microscopy showed that binding of HpD was to mitochondria. Although no tumor cell lines were included in their report, it immediately brought to mind the earlier study by Senior et al. (1975), in which they characterized inner membrane enzymes and transport systems in mitochondria from the R3230AC mammary tumor, a transplantable, hormone-responsive differentiated carcinoma of Fischer rats (Hilf et al., 1965; Hilf, 1967). Mitochondria from tumors were compared with mitochondria from pregnant and lactating mammary glands. They observed that translocation of ADP across the inner mitochondrial membrane was more rapid by virtue of a lowered Km ADP and a raised Vmax, whereas transport of phosphate and dicarboxylic acids

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occurred at similar rates in all three tissue sources. Hence, it was decided that a study of photosensitization of tumor mitochondria might provide mechanistic insight into the actions of HpD.

While many of the general properties of mitochondria from R3230AC tumors were functionally normal, e.g., pyruvate dehydrogenase, Krebs cycle, NADH oxidase, succinate oxidase, respiratory control ratios, P/O ratios and cytochrome oxidase activities, the average yield of mitochondria from tumors was considerably lower than mammary tissues from pregnant or lactating rats (Senior et al., 1975). Based on these findings, we elected to examine in detail the effects of HpD and light on cytochrome c oxidase (EC 1.9.3.1), the terminal oxidant catalyzing electron transfer from cytochrome c to oxygen, a critical step in aerobic metabolism. The initial studies were performed on tumor mitochondria exposed to HpD (and some other porphyrins) *in vitro* (Gibson and Hilf, 1983). Inhibition of cytochrome c (cyt c) oxidase activity was dose-dependent and light dose-dependent (time of light exposure); under the conditions used, a relationship of percentage of inhibition to HpD dose was observed. Further, the log of percentage of inhibition was linear in time. Nevertheless, these data on inhibition of cyt c oxidase activity, although novel in tumor mitochondria, remained to be demonstrated under conditions *in vivo*. To accomplish this, we administered HpD to rats bearing R3230AC tumors, sacrificed the animals at various times after treatment, prepared mitochondria from the excised tumors, and photoirradiated the isolated organelles *in vitro*. Mitochondria obtained at 24, 72 or 96 hr after HpD administration *in vivo* demonstrated similar and significant photosensitization; inhibition of cyt c oxidase activity after 3 hr of light exposure was 81%, 75% and 72%, respectively. These results from our *in vivo-in vitro* experimental protocol clearly indicated that HpD (and some other porphyrins) were taken up and retained by tumor mitochondria *in vivo*. We suggested that this *in vivo-in vitro* protocol offers a very useful assay for examining dose and time course relationships of photosensitizers, an assay that also takes into account any inherent metabolism by the tumor-bearing host and/or target cell.

Because singlet oxygen has a limited migration range from its site of formation (Moan and Berg, 1991), we surmised that its effects on a biochemical parameter would reflect its cellular or subcellular localization. Therefore, we undertook a more systematic investigation of the effects of PDT on selected enzyme activities, the selection being guided by the extensive biochemical literature that reported their subcellular locations by use of more traditional physical fractionation techniques. Subsequent to initially investigating the effects of HpD and light on isolated mitochondria and cytosol *in vitro*, wherein we observed varying degrees of inhibition of enzymes, we turned to the *in vivo-in vitro* protocol. In that study, mitochondria and cytosol from R3230AC mammary tumor-bearing rats were prepared at various times up to one week after a single injection of HpD. Pyruvate kinase was markedly inhibited at early times, returning to initial levels at 48 hr; neither lactate dehydrogenase nor glucose phosphate isomerase were altered by this treatment scheme. In contrast, mitochondrial succinate dehydrogenase and cyt c oxidase activities displayed significant inhibitions, the greatest decreases occurring between 24 and 96 hr after injection of HpD. At one week after treatment, succinate dehydrogenase activity had returned to its initial level but cyt c oxidase activity remained significantly inhibited. We concluded that HpD reached and remained at the inner mitochondrial membrane, where it could be photoactivated and produce damage to enzymes localized at that site (Hilf et al., 1984).

The next mitochondrial enzyme that we investigated was the proton-translocating adenosine triphosphatase (proton ATP-ase), an enzyme performing the critical role of coupling electrochemical proton gradients to formation of ATP (Perlin et al., 1985). As anticipated, sub-mitochondrial particles exposed to HpD and light resulted in a marked inhibition of proton transport, the inhibition displaying both drug-dose and light-dose relationships. Both inhibition of transport and ATP hydrolysis showed an inhibition of 2% per minute. Upon measuring membrane integrity, either by sucrose leakage or K^+ leakage, we concluded that discrete membrane alterations were not the likely cause of the initial loss of gradient formation. Further, photosensitization by HpD to inhibit ATP hydrolysis and proton transport could not be attributed to cross-linking of enzyme subunits. These results, taken together with our earlier reports enabled us to strongly suggest that mitochondrial enzymes involved in oxidative phosphorylation are very important sites of action for the cytotoxicity of PDT.

Although there has been ample agreement that porphyrin photosensitization plus light leads to singlet oxygen production and cytotoxicity, a consensus had not been reached regarding intracellular site(s) of action of PDT. Cellular components that have been implicated are the plasma membrane (Kessel, 1977; Kohn and Kessel, 1979), nucleus (Moan et al., 1980; Gomer, 1980; Gutter et al., 1977; Fiel et al., 1981), and microsomes (Dixit et al., 1983; Das et al., 1985). Our studies had focussed on mitochondria as an important target and we had demonstrated significant dose related inhibitions of the activities of cyt c oxidase, succinate dehydrogenase and the F_0F_1 ATP synthase, effects seen *in vitro* with isolated mitochondria prepared from tumors of animals that had been injected with HpD. From this experimental model we had concluded that the photosensitizer was retained by mitochondria, whereas effects on plasma membrane and cytosol components occurred very early and were no longer demonstrable at longer times (>24 hr). We surmised that susceptibility to HpD plus light was greater for enzymes located in

the more hydrophobic regions than those located in the more hydrophilic sites, i.e., mitochondrial matrix. To extend this supposition, we next examined effects of PDT on adenylate kinase, an enzyme located in the intermembrane mitochondrial space, and monoamine oxidase, an enzyme bound to the outer mitochondrial membrane (Murant et al., 1987). Using the *in vivo-in vitro* protocol and sampling at 2 to 72 hr after administration of Photofrin II (the clinically approved preparation from HpD), photosensitization of monoamine oxidase was observed at 2 hr (30% inhibition) but no significant inhibition of activity was seen at later times. Interestingly, the activity of adenylate kinase, located in the more hydrophilic intermembrane space, was unchanged over the entire time course. Based on results obtained for cyt c oxidase in these preparations, which showed sustained inhibition of enzyme activity, we concluded that neither monoamine oxidase nor adenylate kinase likely contribute to tumor cytotoxicity under the usual protocol conditions of PDT.

Therefore, these data taken together with our previous reports, suggest the following sequence of events wherein tumor cells, exposed to HpD or Photofrin II, initially take up these porphyrin photosensitizers in the plasma membrane (Table 1). Subsequently these porphyrins enter the cytoplasm where they can sensitize enzymes such as pyruvate kinase, and may briefly contact the outer mitochondrial membrane, affecting monoamine oxidase. By 24 hr and later after administration *in vivo*, it appears to be the inner mitochondrial membrane that has concentrated the more hydrophobic porphyrin components. Since the time that most clinical protocols initiate photoradiation, such as 48 to 72 hr after injection of Photofrin II, the components located in or on the inner mitochondrial membrane, cyt c oxidase, F_0F_1 ATP synthase and succinate dehydrogenase, are most susceptible to the deleterious effects of singlet oxygen. If this proposed sequence that is based on the conducted animal experiments is a reflection of the clinical protocols that show anti-tumor efficacy, then those mitochondrial effects that we demonstrated are likely contributors to the mechanisms of PDT-induced cytotoxicity.

Table 1 Time after Photofrin II administration

Enzyme	2 h	24 h
Na^+K^+ ATPase	43.8 ± 4.7	55.6 ± 7.0
MgÂ†+ATPase	92.2 ± 1.8	86.4 ± 2.5
5′-nucleotidase	100 ± 3.4	99.3 ± 3.4
Pyruvate kinase	94.5 ± 3.1	93.8 ± 2.1
Monoamine oxidase	71.8 ± 6.4	86.4 ± 5.5
Adenylate kinase	95.8 ± 1.5	93.7 ± 1.0
Cytochrome c oxidase	67.0 ± 4.0	26.8 ± 1.7
F_0F_1ATPase	62.6 ± 4.5	34.4 ± 4.2
Succinate dehydrogenase	79.2 ± 2.0	39.7 ± 2.7
NADH dehdrogenase	99.5 ± 2.2	72.6 ± 1.7

Note. Tumor-bearing rats were injected i.p. with 25 mg kg^{-1} Photofrin II, mitochondria prepared from tumors at selected times and exposed to 300–400 J cm^{-2} broad band irradiation. Data are presented as percentages of initial enzyme activity (zero time before photoradiation) ± s.e.m. Each number represents the mean of at least four separate experiments performed in duplicate. Data from Na^+K^+ ATPase, Mg^{2+} ATPase and 5′-nucleotidase are from Gibson et al. (1989) and from monoamine oxidase and adenylate kinase are from Murant et al. (1987).

It was evident that mitochondrial enzymes involved in oxidative phosphorylation and electron transport, and thus production of ATP, had to be considered as potentially critical targets of PDT. It was logical to undertake quantitative measurement of ATP levels, anticipating that a reduction in ATP would potentially compromise cell growth and division because of the central role of ATP in energy metabolism. Initially, we examined effects of PDT on dissociated tumor cells, wherein a drug-dose and light-dose dependent decrease in ATP levels was observed (Hilf et al., 1986). Most interesting was the apparent coordinate behavior of ATP levels and cell viability, as estimated by trypan blue exclusion. Because numerous subcellular sites of HpD action have been suggested as leading to cytotoxicity, we needed to consider that cancer cells display increased anaerobic and aerobic glycolysis for energy metabolism. We reasoned that use of inhibitors known to selectively reduce mitochondrial oxidative phosphorylation, such as oligomycin that binds to the F_0 portion of the proton-translocating ATPase, could provide a means to confirm the mitochondrial site of action of HpD photosensitization. Hence, mitochondria treated with oligomycin to reduce ATP levels to 50% of their initial values would not be expected to display further significant reduction in ATP after HpD photosensitization. Those predictions were borne out with very little further reduction in ATP levels by PDT. To distinguish ATP production by glycolysis, we employed iodoacetate, which inhibits glyceraldehyde 3-phosphate dehydrogenase and prevents formation of ATP and NADH. Using a similar experimental design in which the iodoacetate reduced ATP levels by 50%, the activation of HpD by light caused a further reduction of ATP levels to reach <10% of the initial levels. These findings lend further support to our earlier conclusion that the major site of HpD photosensitization is the mitochondrion.

Despite all of the data obtained from studies *in vitro* on isolated mitochondria exposed to PDT, on intact dissociated tumor cells obtained from cell cultures, on intact dissociated tumor cells obtained from tumors growing *in vivo*, and from the *in vivo-in vitro* protocol that we initiated and takes into account the metabolic and pharmacological events that systemic administration of HpD and Photofrin II may undergo, there remained a concern that none of the experimental methods accurately mirrored the events occurring in tumors *in situ* treated with PDT. Fortunately, ^{31}P-NMR enables one to study, under analogous conditions, the photosensitizing ability of systemically administered porphyrins (or other photosensitizers) in the intact tumor-bearing animal.

Table 2 Phosphorus resonance ratios *vs* time between PII injection and Photoradiation

Time after radiation (h)	P_i/Total (%) time after PII			βATP/P_i time after PII		
	0.5 hr	24 hr	72 hr	0.5 hr	24 hr	75 hr
0 (no hv)	7.59	9.03	14.4	2.0	1.61	1.00
1.0	23.6	19.5	23.2	0.35	0.59	0.49
3.0	42.6	28.5	35.5	0.12	0.38	0.20
5.5	50.9	34.2	39.7	0.0	0.13	0.17
24	20.4	24.8	14.1	0.59	0.28	0.46
30	21.7	20.6	10.0	0.76	0.53	1.23

Note. All animals received i.p. 25 mg kg^{-1} PII. Photoradiation conditions were 200 mW cm^{-2} for 1 h, 530–700 nm. Tumors were 0.7–1.2 cm diameter at time of radiation. Data represent results from 2–3 animals treated similarly.

We reported use of this technique in an initial study (Ceckler et al., 1986), where we observed a rapid and dramatic decrease in ATP (NTP) levels in tumors of animals treated with Photofrin II and light. Those results encouraged us to pursue a more extensive series of experiments to help define early responses monitored by NMR (Hilf et al., 1987; Ceckler et al., 1991). In these experiments, we administered Photofrin II (25 mg/Kg) to R3230AC tumor-bearing rats and tumors were exposed to 200 mW/cm^2 for 1 hr (54J of 530–700 nm). As shown in Table 2, the area of the βATP peak relative to the total Pi resonances declined dramatically, reaching 0 to <15% of initial levels when tumors were measured at 5.5 hr after photoradiation. There was a concomitant marked increase in Pi. These effects appear to be fluence related. One possible mechanism for reduced tumor ATP levels after PDT could involve changes in the vascular supply to the tumor. We attempted to measure changes in tumor vascularity by the rate of appearance of administered D_2O. Compared to control, PDT did not appear to alter the uptake of D_2O significantly in treated tumors. At the ultrastructural level, the degree of necrosis after PDT was faster in its development and more extensive than would be expected from impairment of blood flow. Hence, while considerable interest was raised implicating possible damage to vascular endothelial cells as contributing to or causing cytotoxicity, our data obtained in these experiments do not support the notion that reduced ATP (NTP) levels occurred primarily as a result of major changes in tumor vascularity. Taken together with our earlier results, and of others (Chopp et al., 1987), reduction in tumor ATP levels *in situ* is a direct and early response to PDT, most probably a result of inhibition of mitochondrial function.

In sum, our studies leave little doubt that the inner mitochondrial membrane is a target of PDT when the photosensitizer is HpD or its clinically approved mixture of the more active porphyrin oligomers present in HpD, designated Photofrin II. Using a rodent mammary adenocarcinoma as the tumor model, we demonstrated this in isolated mitochondria, in cultured cells and in a protocol referred to as *in vivo-in vitro*, in which the sensitizer was administered to tumor-bearing animals, allowed to accumulate in the neoplasm, and mitochondria were prepared therefrom and exposed to photoradiation. In all of these experiments, marked and significant drug-dose and light-dose dependent inhibition of critical enzymes of oxidative phosphorylation was observed. We demonstrated that this inhibition led to dramatic decreases in cell ATP levels and conclusively proved that such declines after PDT occurred in tumors *in vivo* utilizing the non-invasive NMR technique. We conclude that rapid and significant inhibition of mitochondrial function result from porphyrin photosensitization.

Since the initial report by Oleinick and her colleagues, demonstrating a dose- and time-dependent DNA fragmentation in mouse lymphoma cells sensitized by chloroaluminum phthalocyanine (Agarwal et al., 1991), along with observed chromatin condensation, the implication that apoptosis (programmed cell death) played a mechanistic role in the antitumor effects of PDT had to be explored. Because many of the macrocyclic photosensitizers localize in or associate with mitochondria, and because earlier studies demonstrated the effects of porphyrin photosensitizers on mitochondrial components, together with the fact that mitochondria are a key organelle in many apoptotic pathways, it is not surprising that considerable recent research has focussed on the role of mitochondria in PDT-induced apoptosis. Results of these many investigations regarding mechanisms of apoptosis are thoroughly reviewed and critically evaluated by Oleinick et al. (2002), including the difficulties in demonstrating such events in tumors *in vivo*. Because many of the explorations of PDT-induced apoptosis revolve around a role of mitochondria, these studies continue to support these organelles as important targets of the efficacy of PDT as an anti-neoplastic therapy.

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