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PHOTODYNAMIC INACTIVATION OF RED CELL UROPORPHYRINOGEN DECARBOXYLASE BY PORPHYRINS

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Abstract—1. The effects of light and porphyrins on the activity of red cell uroporphyrinogen decarboxylase were studied.

2. Photoinactivation of uroporphyrinogen decarboxylase was dependent on uroporphyrin concentration, irradiation time and temperature. Using 40 W/m^2 of UV light intensity, 40–45% decreased activity was produced with 200 μM uroporphyrin I, at 37°C and after 2 hr of illumination.

3. It has been demonstrated that porphyrins photoinactivate uroporphyrinogen decarboxylase and a mechanism for this action in relation to skin lesions is proposed.

INTRODUCTION

Solar energy is directly or indirectly responsible for sustaining all forms of life on earth; it is absorbed by plants and converted into chemical energy through the synthesis of carbohydrates, which in turn produce oxygen, critical to cellular metabolism. Central to the photosynthetic process is chlorophyll, a porphyrin containing molecule, while heme, another porphyrin-containing compound, plays a fundamental role in eucaryotic cells respiration. Porphyrins are therefore a basic requirement for aerobic and anaerobic metabolism.

The discovery of photosensitization as a property of porphyrins dates back to the early observations of Anderson (1898), Haussmann (1911) and Meyer-Betz (1913) who suggested that there might be a relation between photosensitivity and enhanced urinary excretion of porphyrins. It is worth noting that porphyrins are the only photosensitizers definitely synthesized internally. Increased cutaneous photosensitivity is a hallmark in the diagnosis of cutaneous porphyrias. In these instances, the vital effect of light and oxygen is changed into a harm one, because porphyrins accumulated in the skin are excited by short-wave UV light to triple state porphyrins, which by energy-transfer produce reactive oxygen, this in turn may oxydize cellular compounds, and thereby initiate damage of the cell which eventually could lead to its death.

On the other hand, accumulation of porphyrins at neoplastic loci had been already reported in 1924 (Policard, 1924) and in the forties (Auler and Banzer, 1942; Figge *et al.*, 1948), but it is Lipson *et al.* (1961) who described porphyrin localization in human tumors following injection of a haematoporphyrin derivative. Today photoactivated porphyrins are being evaluated for their use in cancer therapy (Kelly and Snell, 1976; Daugherty *et al.*, 1978) on the basis of two particular properties of these unique compounds, their tumor localizing ability and their photodynamic action as noted by Diamond *et al.* (1972).

Increased photosensitivity is the most evident clinical sign in symptomatic PCT, due to accumulation of highly carboxylated porphyrins in the skin (Polo *et al.*, 1983), as yet, however, their relative photosensitizing potency and the mechanism(s) of this action still remain a matter of speculation.

Excessive accumulation and excretion of porphyrins is due to a hepatic hypoactivity of uroporphyrinogen decarboxylase (EC 4.1.1.37) (URO-D), in both hereditary and sporadic PCT (De Verneuil *et al.*, 1978), although red cell enzyme deficiency is only detected in the hereditary type, while normal activity is found in the acquired form (De Verneuil *et al.*, 1978; Doss *et al.*, 1980; Elder *et al.*, 1980; Afonso *et al.*, 1985).

It has been shown, that in hexachlorobenzene induced porphyria, uroporphyrins produced endogenously in the cytosol, concentrate in the lysosomes, and it was suggested that photodamage to lysosomes may be of primary importance to explain the cutaneous lesions in PCT (Sandberg and Romslo, 1982). In addition Allison and Young (1984) have found that lysosomes accumulate porphyrins added externally to cells. However it is still unknown what is the subcellular localization of porphyrins in the skin, neither is known what is the origin of the excess skin porphyrins, or what is the inherent sensitivity of the different subcellular organelles to porphyrin induced photodamage (Gschnait *et al.*, 1975). Moreover, data on porphyrin biosynthesis are scarce, but there is some evidence indicating that accumulation of porphyrins in the skin of PCT could be due in part to a decrease activity of URO-D in this tissue (Elder *et al.*, 1981; Polo *et al.*, 1984).

It has been demonstrated that both porphyrins and porphyrinogens, are inhibitors of URO-D (Mauzerall and Granik, 1958; De Verneuil *et al.*, 1980) and it has

been proposed that they might act competitively, occupying the site of the substrate (De Verneuil *et al.*, 1980). We think, that, besides this inhibitory effect, porphyrins might also act at the cellular level, photoinactivating the enzyme. To gain experimental support for this proposal a series of assays have been carried out and preliminary results are reported here.

MATERIALS AND METHODS

Most of the chemicals were obtained from Sigma Chem Co unless stated. Uroporphyrin I (URO I) was generously provided by Dr Torben With from Denmark, uroporphyrin III was either obtained from Porphyrin Products, U.S.A. or prepared from Turacin (With, 1957). The rest of the porphyrins used were from Professor Batlle's stock.

Enzyme source

Fresh human blood was obtained from the blood bank of Ramos Mejía Hospital, Buenos Aires, and whole blood hemolysates prepared as described elsewhere (Batlle *et al.*, 1978) were used. It has been reported that externally added uroporphyrin does not penetrate membranes (Sandberg *et al.*, 1982), so that is one of the reasons why we have employed hemolysates here.

Uroporphyrinogen decarboxylase activity was determined by the method reported by Afonso *et al.* (1985); one unit is expressed as the nmoles of products formed (hepta + hexa + penta + coproporphyrin) from uroporphyrinogen III in 60 min. Specific activity being the number of units per mg of protein.

Irradiation

10 ml of whole blood hemolysate were incubated at varying temperatures in Pyrex glass Petry dishes, of 40 mm diameter immersed in a water bath with a controlled temperature, in the presence of different concentrations of free uroporphyrin I (or other porphyrin as indicated), the final pH of the system was 7.0. Two PUVA lamps (Philips model HPW 125) with maximal emission at 365.5 nm were used, they were placed on either site at 10 cm from the incubation vessels, to obtain a total light intensity as measured at sample level of 40 W/m^2. On a sunny day with sun altitude 70°C, the light intensity in UV-A is approximately 45 W/m^2 (Bickers *et al.*, 1979) and the light intensity reaching the dermis under these conditions should be about 20 W/m^2 (Bickers *et al.*, 1979). Irradiation was carried out in air for different time intervals.

After irradiation the suspension was treated with Dowex 1-X8 (200–400 mesh) anionic resins, following the procedure described elsewhere (Afonso *et al.*, 1985) to separate the porphyrins externally added; after this treatment URO-D was measured in the resulting system. Controls without light and/or porphyrins were also run in all experiments, and the activity of those run in the dark and in the absence of photosensitizer was taken as 100%. All other materials or methods not especified here were those already reported (Afonso *et al.*, 1985; Batlle *et al.*, 1985).

RESULTS AND DISCUSSION

Effect of porphyrin concentration

Taking into account that in human PCT, a great proportion of accumulated and excreted uroporphyrins belongs to series I (Batlle *et al.*, 1985) we decided to carry out these preliminary experiments using URO I as photosensitizer. Keeping both irradiation time and incubation temperature fixed, the concentration of porphyrin was varied. Figure 1 shows that under these conditions enzyme activity is dependent on the amount of porphyrin present in the system during UV irradiation, and inactivation was about 42% at 200 μM URO.

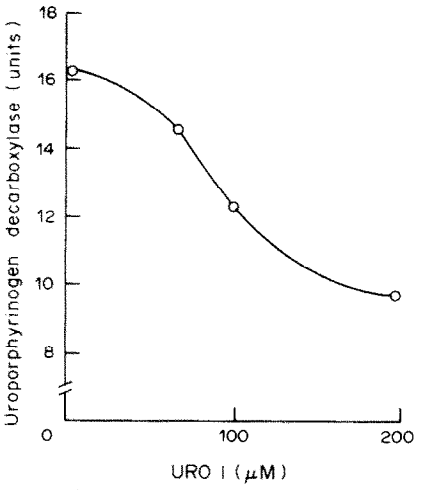

Fig. 1. Effect of different concentrations of URO I and UV light on red cell Uroporphyrinogen decarboxylase. Irradiation time was for 2 hr and temperature was 37°C. Other experimental conditions were as indicated in Material and Methods.

Effect of irradiation time

Another important factor to evaluate the possible photodynamic action of porphyrins on activity, is the irradiation time; it is therefore shown in Fig. 2 that, as expected, there is a linear and inverse relationship between these two parameters; and in agreement with previous results, nearly 45% inactivation was reached after two hours of irradiation in the presence of URO I. This increase in sensitivity to UV light with increasing incubation time might also be related to protein uptake of the photosensitizer. It has also been shown here, that the enzyme is slightly photoinactivated, even in the absence of porphyrin, but obviously very much larger light doses would be

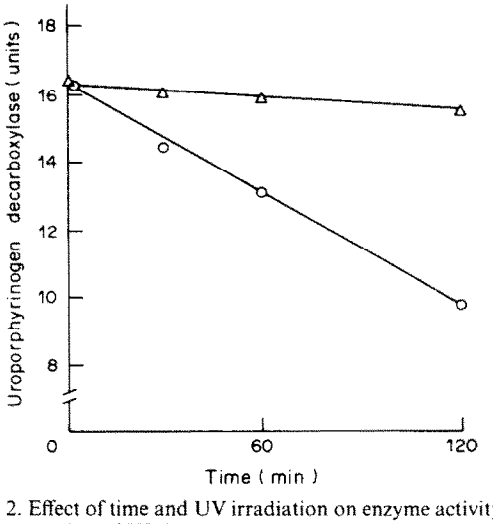

Fig. 2. Effect of time and UV irradiation on enzyme activity. Concentration of URO I used was 200 μM and temperature was 37°C (○). Same conditions but in the absence of URO I (△). Other experimental conditions as indicated in the text.

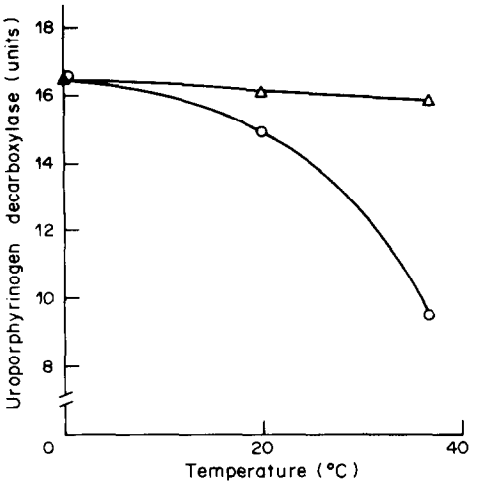

Fig. 3. Effect of temperature on enzyme activity. Irradiation time was for 2 hours and concentration of URO I was 200 μM (○). Same conditions but in the absence of URO I (△). Other experimental conditions as indicated in the text.

necessary to reach the same degree of inactivation as compared to that obtained in the presence of the sensitizer. It is also deducted from these findings that irradiation for longer periods would also have produced greater effect under the light intensity here chosen.

Effect of temperature

The influence of temperature during irradiation it is of great importance to elucidate the kind of changes that are produced on the protein structure by the action of both light and photosensitizer.

Interesting results are illustrated in Fig. 3, where it can be seen that in this case photoinactivation is dependent on incubation temperature, practically no changes were observed at 3°C, only 10% decrease at 20°C and again, 45% at 37°C. Once again it was recorded that URO-D in very slowly photoinactivated in the absence of the porphyrin.

It is accepted that the primary effect of irradiating proteins in the presence of a sensitizer is photooxidation of sensitive amino acid residues, which is considered to be the most possible reason of photodynamic cell inactivation (Verweij *et al.*, 1981; Van Steveninck *et al.*, 1983; Hilf *et al.*, 1984). It has been shown that illumination of spectrin in the pH range 7.0–8.5 in the presence of 50 μM protoporphyrin leads to photooxidation of the sulfhydryl groups of cysteine which are the most sensitive; followed by histidine, tryptophan and tyrosine (Van Steveninck *et al.*, 1983). The same sequence of sensitivity was observed with other proteins. In many cases, however, a secondary reaction between photooxidized aminoacid residues and other groups in the protein molecule leads to interpeptide covalent cross-linking, which can easily be visualized in polyacrylamide gel electrophoresis (Van Steveninck *et al.*, 1983; De Goeij *et al.*, 1975) and similar photodynamic intrapeptide cross-links could also occur (Van Steveninck and Dubbelman, 1984).

Photooxidation and secondary cross-linking are not necessary coupled events.

It has been reported that in the temperature range from 0 to 40°C, photooxidation is practically temperature independent, while the degree of interpeptide cross-linking is significantly decreased at lower temperatures (Van Steveninck *et al.*, 1983), in accordance with the suggestion that cross-linking has a much higher energy of activation than does photooxidation. Accumulated evidence also indicate that cross-linking succeeds photooxidation as a secondary but not compulsory reaction. Photodynamic cross-linking should be caused, therefore, by a secondary reaction between a photooxidized aminoacid residue and reactive groups in the protein molecule, leading to the formation of a covalent bond (Dubbelman *et al.*, 1980) and the most probable mechanisms are based on the interaction of photooxidized histidine with amino, sulfhydryl and imidazol residues of the same or a different molecule; indicating that in proteins exhibiting intermolecular cross-linking, intramolecular cross-linking could also occur (Tomita *et al.*, 1969; Verweij and Steveninck, 1981).

According to the present findings, uroporphyrin produces photoinactivation of red cell URO-D, which is dependent on the concentration of porphyrin, irradiation time and temperature. Therefore this action of uroporphyrin as a photosensitizer would be the result of a photooxidation, very likely of sulfhydryl groups in the protein; we might recall that URO-D is a sulfhydryl enzyme, and these —SH groups are very sensitive to a great number of thiol reagents (Kawanishi *et al.*, 1983; De Verneuil *et al.*, 1983), although photooxidation of other residues might also occur, because 10% of its aminoacids composition is formed by aromatic ones, of which 11 are histidine and 10 tryptophan molecules (De Verneuil *et al.*, 1983). However, in this case URO-D photooxidation is followed by secondary cross-linking, as shown by the temperature studies. Obviously further experiments are needed to better support this proposal.

Effect of different porphyrins

The action of porphyrins other than URO was also tested, at a concentration of URO producing between 20–25% photoinactivation, because the effect of the others could be either much greater or lower than that of URO. It was found (Fig. 4) that PROTO was twice as effective as URO while MESO and COPRO had some but lower action.

Again some photoinactivation of the protein still occurred in the absence of porphyrins. We cannot yet offer a good explanation for the different behaviour of the different porphyrins.

Concluding remarks

Besides the known inhibitory action of the porphyrins on URO-D, we have demonstrated here that these same compounds produce photoinactivation of the enzyme, this photodamage may be of great importance to explain the development of cutaneous lesions in PCT, which are exacerbated in summer time and in light exposed areas (Burnett and Pathak, 1963).

Although extrapolation from experiments *in vitro* to the situation *in vivo* is extremely difficult and should be done with great caution we proposed that

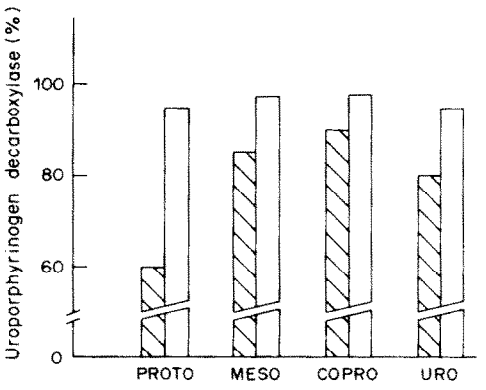

Fig. 4. Photodynamic action of different porphyrins on enzyme activity. The concentration of all porphyrins used was 60 μM, temperature was 37°C and irradiation time was 2 hours. Irradiation in the presence (▨) and in the absence (□) of porphyrin. Other experimental conditions were as indicated in the text.

as a consequence of a primary enzymatic deficiency, in the liver, either hereditary or acquired, porphyrins are accumulated; they are probably taken up from the plasma by endothelial cells of the skin, as proposed by Gschnait *et al.* (1975) and once there would act as photosensitizers, inactivating even more skin URO-D, thus increasing further the local concentration of porphyrins. When the amount of porphyrins in the skin reaches certain level; they might now play its photodynamic action on the lysosmes with release of lysosomal enzymes and finally production of the typical cutaneous lesions. It is interesting to remember that 20 years ago, Burnett and Pathak (1963) had already observed that in PCT patients, exposure of the skin to radiant energy produced an increased formation of uroporphyrin and increased urinary and fecal excretion of this porphyrin and Bickers *et al.* (1977) have found that cultures from fibroblasts from PCT patients synthesize significantly more porphyrins than fibroblasts from healthy controls. Therefore, porphyrin sensitized photodynamic inactivation of URO-D and damage of other biomolecules and lysosomes in the skin, are the primary events that end in the cutaneous abnormalities characteristics of PCT.

Porphyrins would than have the dual property of inhibiting and photoinactivating URO-D, extending its photodynamic action to lysosomal damage.

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