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PORPHYRIN-SENSITIZED PHOTODYNAMIC DAMAGE OF ISOLATED RAT LIVER MITOCHONDRIA

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Summary

The respiration rates and the respiratory control ratios of isolated rat liver mitochondria have been measured following exposure to 0—160 kJ/m^2 of near-ultraviolet radiation (blacklight) in the presence of low concentrations of porphyrins (0.1—0.2 μmol/l).

Depending on the light dose, the concentration and the type of porphyrin, the following sequence of reactions occurred: uncoupling and inhibition of oxidative phosphorylation, energy dissipation, inhibition of respiration and swelling and disruption of the mitochondria.

The detrimental effects could not be elicited in the absence of oxygen, neither could they be elicited by porphyrins or light alone.

At equimolar concentrations, the effectiveness of the porphyrins as photosensitizers were: deuteroporphyrin > protoporphyrin >> coproporphyrin > urophorphyrin.

The results may be of importance to explain the skin lesions seen when porphyrins of different hydrophobicity accumulate in the skin.

Introduction

Cutaneous photosensitivity is a prominent symptom in a number of porphyrias [1]. The skin lesions are probably produced by free oxygen radicals [2,3] or singlet oxygen [3,4] generated by energy-transfer from photoexcited

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCIP, dichlorophenolindophenol.

porphyrins accumulated in the skin [5,6]. The porphyrin-sensitized photodynamic lesions have been studied in multicellular organisms, cell organelles, biomacromolecules and small organic molecules (for review, see Spikes [5]). In red cells, photooxidation of amino acids and photoaggregation with evidence of crosslinking of membrane polypeptides have been noted [7—9]. In the skin, however, the metabolic derangements which follow exposure to the active oxygen species are poorly understood [10,11].

Porphyrins with two carboxylic groups, but not porphyrins with four or more carboxylic groups, are potent uncouplers of oxidative phosphorylation [12—14]. Uncoupling of oxidative phosphorylation in the same fashion as in liver mitochondria, however, can hardly explain the cutaneous lesions seen when porphyrins accumulate in the skin, because the amount of porphyrins is too small for uncoupling, i.e., about 100—200 nmol/l protoporphyrin [15,16], and also because skin lesions are found when uro- and coproporphyrins accumulate [1,16].

Irradiation of isolated mitochondria induces uncoupling [17—22] inhibitions of respiration [17,20,21] and swelling and disruption of the particles [22,23]. The sensitivity to injury increases from visible light to far-ultraviolet light by a factor of about 100 000 [20,21] and it is markedly increased by photosensitizers like FMN and riboflavin [21]. Also the inhibition of mitochondrial respiration by copper- and sodium-chlorophyllins is markedly increased by light [24].

It could be speculated that porphyrins when irradiated with near-ultraviolet light may potentiate the photodamage to the mitochondria even at the minute porphyrin concentrations found in the skin [15,16], and if the resulting mitochondrial dysfunction may be a decisive factor to the cutaneous lesions in some of the porphyrias. Experiments were therefore undertaken to study in more detail the effect of low concentrations of porphyrins and irradiation on mitochondrial metabolism, using liver mitochondria as the model organelle.

Materials and Methods

Preparation of mitochondria

Rat liver mitochondria were prepared as described [25]. The functional integrity of the preparations was tested by measuring the respiratory control ratio with ADP using succinate as the substrate [25]. Only mitochondria with a respiratory control ratio with ADP of greater than 3 were used.

Irradiations of the mitochondria

Mitochondria of approx. 1.5 mg protein were incubated at +4°C in a final volume of 3 ml: 50 mmol/l glucose, 175 mmol/l sucrose, 5 mmol/l $MgCl_2$, 10 mmol/l KCl, 5 mmol/l P_i and 10 mmol/l Hepes buffer, pH 7.40. Further additions were as indicated in the legends to the figures. The suspension was kept in a test tube of diameter 10 mm and irradiated with a photochemotherapy unit PUVA 200 (H. Haldmann, D 722 Schwenningen, F.R.G.) containing 14 fluorescence tubes (F8 T5/BL PUVA Sylvania) in a bank. About 70% of the emission energy of these lamps is between 340—380 nm. The light dose as measured at a distance of 15 cm from the lamp with an UDT model

80 X optometer equipped with an UDT-1116 filter optic probe (United Detector Technology, Inc., Santa Monica, CA, U.S.A.) was 66 W/m^2. Following irradiation, the suspension was transferred without delay to the chamber of the oxygraph or the cuvette used for the safranine stacking experiments (see below).

In some experiments irradiation was done in the absence of oxygen. Mitochondria were incubated in a Thunberg tube in the medium described above at 25°C. 5 mmol/l succinate was added. The tube was repeatedly evacuated and refluxed with nitrogen (purity greater than 99.99%). Following treatment with nitrogen, the tube was left in the dark for 5 min to secure removal of residual traces of oxygen (by the respiratory activity of the mitochondria). The sample was then irradiated as described above.

Other analytical procedures

Mitochondrial respiration rates were determined in the suspension of the irradiated mitochondrial with a Clark oxygen electrode (Yellow Springs Instrument's Biological Oxygen Monitor) with appropriate polarization circuitry.

The spectral shift of safranine was determined by adding 20 μmol/l safranine to the irradiated mitochondrial suspension. Further additions and experimental conditions were as described (see Fig. 1). The spectra were recorded on an Aminco DW-2 UV/VIS spectrophotometer using the wavelength pair 524—554 nm [12] at 25°C.

Mitochondrial swelling was determined by incubating the mitochondria of approx. 0.5 mg protein/ml in a medium of 0.25 mol/l sucrose/10 mmol/l Hepes buffer, pH 7.40. The suspension was irradiated as described above and the change in absorbance at 520 nm was recorded.

Glutamate dehydrogenase (EC 1.4.1.3) was determined as described previously [26], succinate-phenazinemethosulphate oxidoreductase (EC 1.3.99.1) as described by Arrigone and Singer [27] and adenylate kinase (EC 2.7.4.3) as described previously [28]. Prior to the enzymic assays, the mitochondrial suspension was sonicated for three periods of 30 s in a chamber maintained at +4°C, using an MSE 150 W ultrasonic disintegrator, model MK2, operated with a 9.5 mm diameter probe and an amplitude reading of 18 μm.

Protein was determined by the Folin-Ciocalteau reagent [29].

Chemicals

ADP, ATP, carboxyl cyanide *m*-chlorophenylhydrazone (CCCP), 2,6-di chlorophenolindophenol, phenazine methosulphate, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (Hepes), uroporphyrin I (free base) and coproporphyrin I (dihydrochloride) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Safranine was purchased from Merck AG, Darmstadt, F.R.G. Protoporphyrin IX and deuteroporphyrin IX (crystalline dihydrochloride) were from Porphyrin Products, Logan, UT, U.S.A. The purity of the porphyrins was better than 95% as assessed by thin-layer chromatography on silica gel after esterification [30]. Other chemicals were of the highest purity available commercially. Double-quartz distilled water was used throughout.

Results

Effect on the respiration rate and on the energy state of the mitochondria

When rat liver mitochondria were incubated with deuteroporphyrin under aerobic conditions and exposed to increasing doses of light of 340—380 nm (blacklight), a sequence of reactions occurred. At light doses of 2 kJ/m^2, the State 4 respiration rate (with succinate as substrate) was markedly increased, and concommitantly there was a decrease in the ADP-stimulated respiration rate (State 3) (Table I). By increasing the light dose (20 kJ/m^2) the respiratory control was lost and the State 4 respiration rate was markedly decreased. On the other hand, when the mitochondria were irradiated at 20 kJ/m^2 under anaerobic conditions, or without porphyrin, the State 4 respiration rate and the respiratory control ratio with ADP remained essentially as in the dark control mitochondria.

The light-dose response curves for the State 4 and State 3 respiration rates are shown in Fig. 1. State 4 respiration rate increased about 2-fold at an irradiation of 5 kJ/m^2, but it did not reach the rate obtained in State 3 respiration of the control mitochondria. At higher light doses, the State 4 respiration rate was gradually inhibited. By contrast, the State 3 respiration rate was inhibited by all light doses. Essentially similar results were obtained when irradiation was kept constant but the concentration of deuteroporphyrin was varied. Thus, at a light dose of 20 kJ/m^2 loose-coupling and inhibition of State 4 respiration were found at deuteroporphyrin concentrations greater than 20 nmol/l and greater than 50 nmol/l, respectively. For comparison, when analogous experiments were run in the dark, uncoupling was found at concentrations of deuteroporphyrin greater than 2—5 μmol/l [12].

Of the many probes used to estimate the energy state of the mitochondrial inner membrane, measurement of the stacking of safranine is one of the more well-established methods [31]. Thus, under conditions of small variations in the transmembrane pH gradient, the energy-dependent stacking of safranine closely parallels the membrane potential.

The light-dose response curve for the change in safranine stacking is shown in Fig. 1. Whereas the respiration rate changed at light doses > 0.7 kJ/m^2, the stacking of safranine showed only minor changes up to light doses of 5 kJ/m^2.

TABLE I

EFFECT OF IRRADIATION AND PORPHYRIN ON THE RESPIRATION RATES OF ISOLATED RAT LIVER MITOCHONDRIA

Mitochondria were incubated and irradiated under anaerobic or aerobic conditions (see Materials and Methods). The concentration of deuteroporphyrin was 180 nmol/l. The State 4 and State 3 respiration rates were initiated by adding 5 mmol/l succinate and 7 mmol/l ADP. The results are the means and the ranges (in parentheses) from three separate experiments.

Irradiation (kJ/m^2)	State 4 respiration rate (nmol oxygen/mg protein per min)	State 3 respiration rate (mol oxygen/mg protein per min)
0 (dark control)	9 (6—12)	37 (31—52)
2	13 (9—15)	24 (22—28)
30	6 (4—8)	6 (4—8)
20, under anaerobic conditions	9 (7—13)	34 (29—41)
20, without porphyrin	11 (10—13)	38 (30—45)

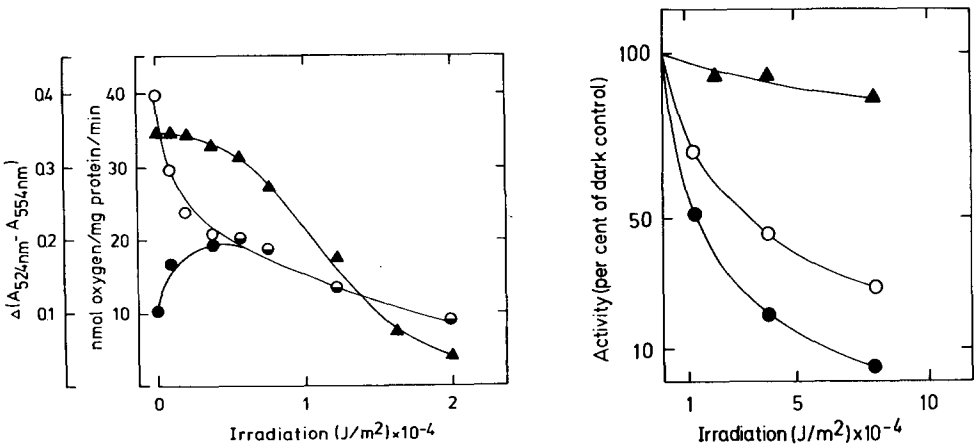

Fig. 1. Effect of irradiation on the energy-dependent stacking of safranine and on the respiration rate of rat liver mitochondria. Mitochondria were incubated and irradiated as described (see Materials and Methods). The concentration of deuteroporphyrin was 120 nmol/l. Following irradiation, one portion of the mitochondrial suspensin was removed for determination of the energy-dependent stacking of safranine (▲), and one portion was removed for determination of State 4 (●) and State 3 (○) respiration rate.

Fig. 2. Effect of increasing light doses on State 4 respiration rate of rat liver mitochondria. Mitochondria were incubated and irradiated as described (see Materials and Methods) in the presence of 100 nmol/l deuteroporphyrin. The substrates were 5 mmol/l succinate (●), 5 mmol/l glutamate + 5 mmol/l malate (○) and 5 mmol/l ascorbate + 100 μmol/l TMPD (▲).

At higher light doses there was a progressive decline in safranine stacking, to reach zero level at about 40 kJ/m^2 (not shown).

The sensitivity of the various respiratory components to inactivation by light, oxygen and deuteroporphyrin differs. Cytochrome oxidase (ascorbate + TMPD) was not significantly affected by light doses up to 80 kJ/m^2 (Fig. 2). By contrast, 50% inhibition was found at 10 and 50 kJ/m^2 for the oxidation of succinate and glutamate, respectively (Fig. 2).

Effect of the structural integrity of the mitochondria

To assess the effect of irradiation and deuteroporphyrin on the structural integrity of the mitochondria, swelling and release of marker enzymes were determined (Fig. 3).

Swelling of the mitochondria was determined from the decrease in absorbance at 520 nm. At concentrations of 120 nmol/l, deuteroporphyrin had no effect on the swelling of the mitochondria in the dark. Following irradiation, however, a marked decrease in absorbance occurred, but only with light doses above 20 kJ/m^2. The light dose necessary to initiate swelling was very close to that necessary to abolish the membrane potential (Fig. 1). When deuteroporphyrin was omitted, or replaced by equimolar concentrations of uroporphyrin (see below), the mitochondria did not swell upon irradiation.

The release of adenylate kinase and glutamate dehydrogenase from the mitochondria is shown in Fig. 3. Adenylate kinase was released to the medium in a curvilinear fashion, being particularly evident at light doses of 40 kJ/m^2. A similar, but less pronounced release pattern was found with glutamate dehydro-

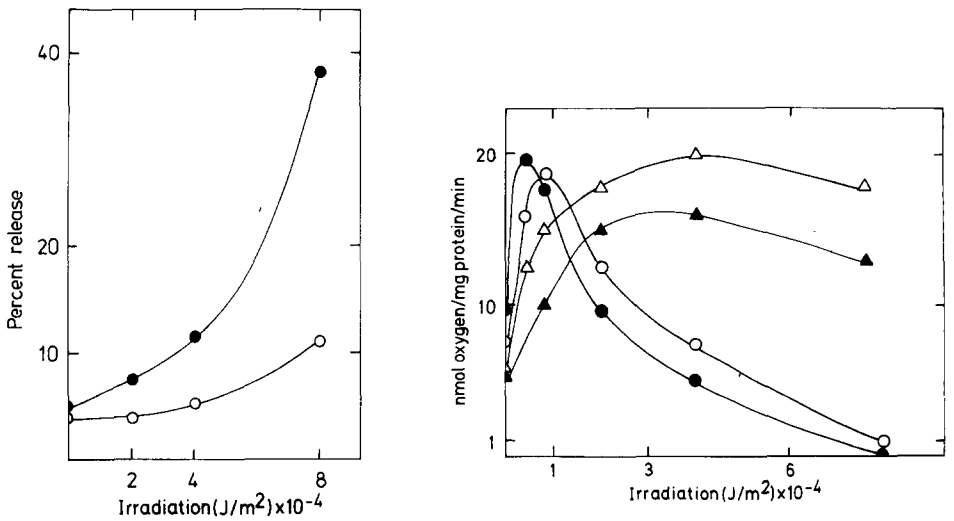

Fig. 3. Effect of irradiation on the release of adenylate kinase (●) and glutamate dehydrogenase (○) from rat liver mitochondria. Mitochondria were incubated as described (see Materials and Methods). The concentration of deuteroporphyrin was 120 nmol/l. Following irradiation the mitochondria were centrifuged at 13 000 × *g* for 2 min. The enzymic activities were determined in the supernatant. 100% activity represents the enzymic activity of the mitochondrial suspension after ultrasonic treatment.

Fig. 4. Effect of uroporphyrin (▲), coproporphyrin (△), protoporphyrin (○) and deuteroporphyrin (●) on State 4 respiration rate of rat liver mitochondria. Experimental conditions were as in Fig. 2. The concentration of the porphyrins was 80 nmol/l.

genase. At 80 kJ/m^2, the release was 10 and 40% for glutamate dehydrogenase and adenylate kinase, respectively.

Effect of protoporphyrin, coproporphyrin and uroporphyrin

So far, all experiments were performed with deuteroporphyrin as the photo-

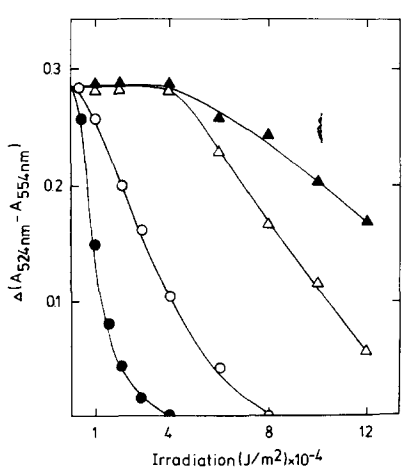

Fig. 5. Effect of uroporphyrin (▲), coproporphyrin (△), protoporphyrin (○) and deuteroporphyrin IX (●) on the energy-dependent stacking of safranine in irradiated mitochondria. Experimental conditions were as in Fig. 1. The concentration of porphyrins was 80 nmol/l.

sensitizer. From a physiological point of view, it is of importance to know what is the effect of porphyrins of human origin. A major difference between the various porphyrins occurring in man concerns their solubility. Protoporphyrin tends to concentrate in lipophilic environments and is excreted in the bile and feces [1]. By contrast, uroporphyrin (and to a lesser extent coproporphyrin) concentrates in hydrophilic environments, and it is excreted predominantly in urine [1]. From this point of view, it would not be unexpected that the effect of the various porphyrins may differ, as actually found (Figs. 4 and 5). Protoporphyrin behaved essentially as deuteroporphyrin with respect to uncoupling, inhibition of succinate oxidation and stacking of safranine. On the other hand, uroporphyrin and coproporphyrin although effective as uncouplers (stimulation of State 4 respiration — see Fig. 4) did not inhibit succinate oxidation and the effect on safranine stacking was found only at light doses greater than 40 kJ/m^2 (Fig. 5).

Discussion

The results show that porphyrins are potent photosensitizers with marked detrimental effects on mitochondria at very low radiation energy in near-ultraviolet. The efficiency of the porphyrins decreases in the order: deuteroporphyrin > protoporphyrin >> coproporphyrin > uroporphyrin.

The sensitivity of the various mitochondrial components to photodamage differs, but the sequence of injuries is essentially as found when mitochondria were irradiated in the absence of porphyrins [21—23].

The photodamage is dependent on oxygen. This suggests a type II mechanism for the photosensitizing process [32]. From studies in other systems, it has been shown that the phototoxicity of porphyrins is due to the generation of singlet oxygen [3,4,33—35] with the possible contribution of superoxide [2,3].

Mitochondria maintain a fairly high membrane potential even at a very low degree of energy coupling [12]. In agreement herewith and also with the data presented by Aggarwal et al. [21] are the results presented in Fig. 1.

The photodamage increases with decreasing wavelength of the incident light. In the experiments of Aggarwal et al. [21], uncoupling of oxidative phosphorylation was found at 5—10 · 10^4 kJ/m^2 when irradiated with light greater than 400 nm. The corresponding figure for uncoupling with light of wavelengths less than 300 nm was 20 kJ/m^2 [22], and with germicidal light (254 nm) mitochondria were uncoupled at 0.9 kJ/m^2 [20]. In our experiments with about 70% of the emission energy between 340 and 380 nm, 20 nm outside the Soret maxima of the porphyrins, uncoupling was found at 1—5 kJ/m^2. In comparison, the near ultraviolet-light dose in 1 min on a sunny day is about 3 kJ/m^2 [36]. It is likely that the photodamage of the porphyrins would be increased if the experiments were run with equivalent radiation energy at the Soret maxima unless a shift in the excitation maxima of the porphyrins is induced by endogenous chromophores of the mitochondria [37]. Also the bandwidth needs to be considered.

Swelling and uncoupling of mitochondria exposed to light in far ultraviolet is well known [22,23], and this ultimately leads to disruption of the

particle [23]. With comparable light doses at near-ultraviolet, no swelling occurs. When deuteroporphyrin is added, the mitochondria swell and release adenylate kinase and glutamate dehydrogenase (Fig. 3).

It is not known what is the biological target for the porphyrin-sensitized photodamage. According to Dubbelman et al. [38], the porphyrin-sensitized photohemolysis of red cells results from a secondary reaction between free amino groups of membrane proteins and photooxidation products of histidine, tyrosin and tryptophan. A similar mechanism has been proposed to explain the lethal effect of near-ultraviolet-light on mammalian cells in culture [39]. The significance of the decline in sulphydryl groups [40], lipid peroxidation [8,40—42] and inactivation of catalase [43] seen during the photodamaging process, is not clear [43].

The effectiveness of the various porphyrins as photosensitizers varies considerably (Figs. 4 and 5). According to Jori et al. [44] two factors seem to be of decisive importance to the toxicity of the porphyrins: the presence and the nature of a complexed metal ion, and the type of the side chain attached to the corners of the pyrrol ring. According to Lamola [45] the solubility and the localization of the porphyrin to lipophilic or hydrophilic environments is an important factor to determine its photo-damaging properties. This asumption is supported by the finding that the lifetime of singlet oxygen in a hydrophobic medium is longer than in an aqueous medium [34, 46]. Our results are in agreement with these findings. Thus, protoporphyrin and deuteroporphyrin induce more severe photodamage to the lipoprotein-rich inner mitochondrial membrane than the more hydrophilic uro- and coproporphyrins. The results are open for some speculations on the pathogenesis of the skin lesions seen in the porphyrias. The skin lesions in erythropoietic protoporphyria differ from those in porphyria cutanea tarda [1] as do the porphyrin species which accumulate [1,15,16]. Thus, in erythropoietic protoporphyria, protoporphyrin accumulates in several organs including the skin [1], whereas in porphyria cutanea tarda uro- and coproporphyrin accumulate [1]. Morphologically, an early finding in erythropoietic protoporphyria is damage to the endothelial cells with swelling and loss of mitochondria [11,47]. On the other hand, in porphyria cutanea tarda the skin lesions are characterized by subdermal oedema, pericapillary fibrillar deposits and normal endothelial cells [11]. Could the lesions be explained by photodamage to hydrophilic and hydrophobic structures depending on the porphyrin species which accumulate? The answer, as yet, is not known.

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