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DESTRUCTION OF MICROSOMAL CYTOCHROME P-450 BY REACTIVE OXYGEN SPECIES GENERATED DURING PHOTOSENSITIZATION OF HEMATOPORPHYRIN DERIVATIVE

RAKESH DIXIT, HASAN MUKHTAR and DAVID R. BICKERS*
Department of Dermatology, Case Western Reserve University and Veterans Administration Medical Center, 10701 East Boulevard, Cleveland, OH 44106, USA

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Abstract—Rat liver microsomal cytochrome P-450 undergoes rapid destruction in the presence of hematoporphyrin derivative (HpD) and solar radiation (~400 nm). Destruction of cytochrome P-450 is associated with the formation of cytochrome P-420 and significant loss of microsomal haem. Quenchers of singlet oxygen including 2,5-dimethylfuran, histidine, β-carotene, and ascorbic acid and inhibitors of the hydroxyl radical such as benzoate, mannitol, and ethanol prevent deterioration of the microsomal haem-protein, whereas superoxide dismutase and catalase are ineffective in this regard. These results indicate that generation of singlet oxygen during hematoporphyrin photosensitization is associated with destruction of microsomal cytochrome P-450 and haem.

INTRODUCTION

The reduction of molecular oxygen in the cell often generates reactive oxygen intermediates including superoxide anion, hydroxyl radicals, and hydrogen peroxide (Kellogg and Fridovich, 1975; Bus and Gibson, 1979). These chemical moieties are relatively unstable, have high biological reactivity, and are potentially toxic. In addition, certain chemicals known as photosensitizers, following exposure to radiation, can absorb energy and transfer that energy to oxygen, thereby generating singlet oxygen, an oxidant with high biological reactivity. One such category of photosensitizing chemicals includes hematoporphyrin (Spikes and Straight, 1967). A modified form of hematoporphyrin known as hematoporphyrin derivative (HpD)† is being used increasingly by clinical investigators in photoradiation therapy for a variety of malignant tumors (Dougherty *et al.*, 1978; Forbes *et al.*, 1980). Several lines of evidence clearly indicate that singlet oxygen generated by hematoporphyrin excited by radiation (~400 nm) results in the oxidation of essential cellular components including lipids, proteins, amino acids and nucleotides (Nilsson and Kearns, 1973; Bodaness and Chan, 1977). Singlet oxygen has also been shown to be associated with cross-linking of membrane proteins, inhibition of the catalytic activity of membrane-bound enzymes, interruption of membrane transport, cell death (Girotti, 1975, 1976; Kahn and Kasha, 1970; Kessel, 1977), photocarcinogenicity (Kahn and Kasha, 1970) and mutagenicity (Gruener and Lockwood, 1979).

Haem-proteins are indispensable constituents of living cells. Among cellular haem-proteins, cytochrome P-450 is unique because of its broad substrate specificity and the biological reactivity of a number of its metabolites (Conney, 1967; Lu and Levin, 1974; Guengerich, 1979). Cytochrome P-450 catalyzes the biotransformation of myriad foreign chemicals including drugs, carcinogens, as well as endogenous substrates including steroid hormones, bile acids and cholesterol (Peterson and Holtzman, 1980). Radiation (~400 nm) of certain haem-proteins, e.g. cytochrome *c*, in the presence of hematoporphyrin results in singlet oxygen mediated destruction of photooxidizable amino acids (Jori *et al.*, 1970, 1971). The studies described in this report were designed to determine whether photosensitization with HpD has any effects upon microsomal monooxygenase activities. Our results provide evidence to show that cytochrome P-450, a major haem-protein of hepatic microsomal membranes, is destroyed by reactive oxygen species generated during photosensitization by HpD.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats (100 ± 10 g) were obtained from Holtzman Rat Farm, Madison, WI, USA. Washed liver microsomes were prepared according to conventional methods (Mukhtar *et al.*, 1978). The twice washed microsomal pellet was suspended in 0.1 *M* potassium phosphate pH 7.4. Protein concentration was determined according to Lowry *et al.* (1951) and the final microsomal pellet was brought to a concentration of 2 mg protein/mℓ. Photooxidation was performed using a Blak Ray long wave UV lamp (Model B-1000A) which has an emission spectrum between 380 and 420 nm; light intensity was determined using a Blak Ray long wave UV meter (Ultraviolet Products, Inc.). A typical reaction mixture consisted of 2.0 mℓ total incubation volume, including 0.1 *M* potassium phosphate pH 7.4, microsomal protein (4 mg)

* To whom correspondence should be addressed.

† *Abbreviations used*: 2,5-DMF, 2,5-dimethylfuran; HpD, hematoporphyrin derivative.

and the desired concentration of HpD. The reaction mixture was exposed to the light source at an intensity of 50 W/m^2 for 5 min at 0–4°C (reaction vessel was immersed in crushed ice throughout the incubation). Cytochrome P-450 content in the microsomal incubation mixtures was determined by the carbon monoxide difference spectrum as described by Omura and Sato (1964). The difference spectrum of carbon monoxide plus dithionite reduced minus dithionite reduced microsomes was recorded in a DW-2a dual beam spectrophotometer (American Instrument Co., Silver Spring, MD) to which a MIDAN microprocessor in the baseline correction mode was attached. An extinction coefficient of 91000 cm^{-1} M^{-1} was used between 450 and 490 nm to determine the cytochrome P-450 (Omura and Sato, 1964) concentration. The entire experiment was conducted in a dark room to obviate the role of extrinsic radiant energy. Microsomal haem and cytochrome b_5 contents were quantitated essentially as described by Omura and Sato (1964). All enzymes and other chemicals were obtained in the purest form from Sigma Chemical Co., St. Louis, MO, USA. Hematoporphyrin was obtained from Porphyrin Products, Logan, UT, USA and HpD prepared according to the method of Lipson *et al.* (1961).

RESULTS AND DISCUSSION

Exposure of microsomes to Soret band radiation ~400 nm in the presence of HpD caused rapid destruction of cytochrome P-450 as measured by difference spectroscopy (Fig. 1). This destructive reaction required both light and HpD since either of these alone had no detectable effect upon the haem-protein (Table 1). The photocatalyzed destruction was oxygen-dependent since exposure of microsomes to HpD and light in a nitrogen-saturated atmosphere did not result in appreciable degradation of cytochrome P-450 (only 10–15% destruction of cytochrome P-450 occurred when the incubation medium was saturated with nitrogen). The decrease in cytochrome P-450 levels was dependent upon the dose of light and the concentration of HpD (Fig. 1A, B). Up to 84% destruction of the cytochrome P-450 occurred in microsomes exposed to 100 kJ/m^2 of radiant energy in the presence of HpD. The decrease in spectrally detectable cytochrome P-450 by photosensitization with HpD was accompanied by an increase in absorbance at 420 nm, suggesting that photodegradation led to the formation of cytochrome P-420, an inactive form of cytochrome P-450 (Murakami and Mason, 1967).

There was a significant loss of microsomal haem content along with the destruction of cytochrome P-450; however, cytochrome b_5 content remained unaltered (Table 1). This further indicates that the destruction of cytochrome P-450 is associated with the loss of haem content by reactive oxygen species generated during HpD photosensitization.

Prior studies have shown that irradiation (Soret band of 400 nm) of HpD in an aerobic medium generates singlet oxygen (Nilsson and Kearns, 1973; Bodaness and Chan, 1977). We, therefore, considered the possibility that singlet oxygen generated by photosensitization of HpD may mediate degradation of cytochrome P-450. To test this hypothesis, the effects of several known quenchers of singlet oxygen were assessed (Table 1). 2,5-Dimethylfuran (2,5-DMF) reacts with singlet oxygen with a second order rate constant of $1.4 \times 10^8 M^{-1}s^{-1}$ (Foote *et al.*, 1967; Foote, 1967). The addition of 2,5-DMF (5 mM) to the complete system of microsomes afforded substantial protection against the photocatalyzed destruction of cytochrome P-450 (Table 2). Thus, the level of cytochrome P-450 in the 2,5-DMF-treated microsomes was 85% of that in the control system and more than 3-fold higher than that seen following HpD and light treatment.

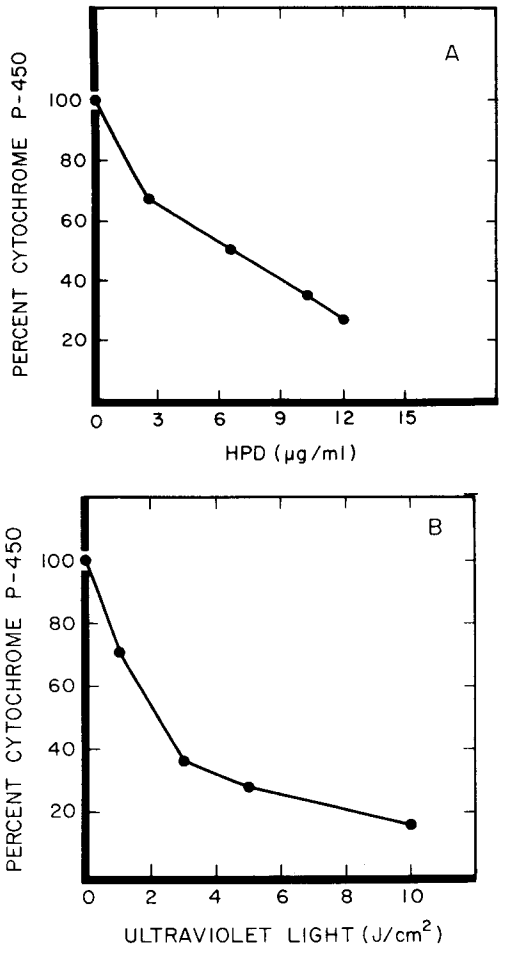

Figure 1. Photodynamic destruction of rat liver cytochrome P-450 by HpD and light (~400 nm). (A) Dependence on HpD concentration: a typical reaction mixture consisted of 2.0 mℓ total incubation volume including, 0.1 M phosphate buffer pH 7.4, microsomal protein (4 mg) and a desired concentration of HpD. The reaction mixture was exposed to the light source at an intensity of 50 kJ/m^2 at 0–4°C (reaction vessel was immersed in crushed ice throughout the incubation). (B) Dependence on light exposure: the experiment was performed exactly as described under (A) with the following exceptions: (1) concentration of HpD used was 12 μg/mℓ, (2) the light intensity was varied. Other details are provided in Materials and Methods.

Table 1. Destruction of cytochrome P-450 and haem by HpD and light (~400 nm)

Incubation system	Cytochrome P-450*	Cytochrome b_5 (nmol/mg protein)	Haem
Microsomes	0.48 ± 0.03	0.50 ± 0.05	0.84 ± 0.09
Microsomes + light (50 kJ/m^2)	0.44 ± 0.05	0.47 ± 0.06	0.78 ± 0.06
Microsomes + HpD (12 μg/mℓ)	0.42 ± 0.06	0.46 ± 0.03	0.81 ± 0.05
Microsomes + HpD (12 μg/mℓ) + light (50 kJ/m^2)	0.10 ± 0.01	0.48 ± 0.07	0.23 ± 0.08
Microsomes + HpD (12 μg/mℓ) + light (100 kJ/m^2)	0.06 ± 0.002	0.50 ± 0.03	0.16 ± 0.03
Microsomes + HpD (12 μg/mℓ) + light (120 kJ/m^2)	0.02 ± 0.001	0.50 ± 0.04	0.10 ± 0.01

Rat liver microsomes (2 mg/mℓ in 0.1 *M* phosphate pH 7.4) were incubated with the following incubation system at 0°C and were exposed to light or dark. Cytochrome P-450 was measured by recording the CO difference spectra of dithionite reduced microsomes as described in Materials and Methods. Haem content and cytochrome b_5 levels were quantitated essentially by the methods of Omura and Sato (1964).

* Data represent mean ± SD of 3 experiments. The amount given in parentheses indicates the final concentration of each in the incubation medium.

Table 2. Effect of scavengers of singlet oxygen on destruction of cytochrome P-450 by UV light and HpD

Incubation system	Cytochrome P-450* (nmol/mg protein)
Microsomes (dark)	0.47 ± 0.05
Complete†	0.12 ± 0.01
Complete + 2,5-DMF (5 m*M*)	0.40 ± 0.06
Complete + 2,5-DMF (10 m*M*)	0.42 ± 0.05
Complete + histidine (5 m*M*)	0.37 ± 0.02
Complete + histidine (10 m*M*)	0.40 ± 0.03
Complete + β-carotene (0.2 m*M*)	0.24 ± 0.02
Complete + ascorbate (5 m*M*)	0.28 ± 0.04

Rat liver microsomes were incubated with the following incubation systems and were exposed to light (50 kJ/m^2). Cytochrome P-450 was measured by recording the CO difference spectra of dithionite reduced microsomes.

* Data represent mean ± SD of 3 experiments.

† Complete = microsomes + HpD (12 μg/mℓ) + light (50 kJ/m^2).

Table 3. Effect of superoxide dismutase, catalase, and scavengers of hydroxyl ion on the destruction of hepatic cytochrome P-450 by UV light and HpD

Incubation system	Cytochrome P-450* (nmol/mg protein)	%
Microsomes (dark)	0.47	100
Complete†	0.12	25.0
Complete + SOD (50 μg/mℓ)	0.13	28.0
Complete + SOD (200 μg/mℓ)	0.14	29.7
Complete + catalase (100 μg/mℓ)	0.14	29.7
Complete + catalase (200 μg/mℓ)	0.13	28.0
Complete + benzoate (5 m*M*)	0.31	67.0
Complete + mannitol (5 m*M*)	0.22	48.0
Complete + EtOH (5 m*M*)	0.22	48.0

Rat liver microsomes (2 mg/mℓ) were incubated with the following incubation systems and were exposed to UV light (50 kJ/m^2). Cytochrome P-450 was measured by recording the CO difference spectra of dithionite reduced microsomes as described in Fig. 1.)

* Data represent values from a typical experiment repeated 3 times with similar values. The amount given in parentheses indicates the final concentration of each in the incubation system.

† Complete = microsome + HpD (12 μg/mℓ) + light (50 kJ/m^2).

Histidine, β-carotene, and ascorbic acid, also known to be effective quenchers of singlet oxygen (Bodaness and Chan, 1979), depressed the photocatalytic degradation of cytochrome P-450 by 25, 53, and 22%, respectively. The spectrally detectable level of cytochrome P-450 in hepatic microsomes was not changed by the addition of these chemicals alone. In further studies we evaluated the possible involvement of superoxide anion, hydroxyl radical, and hydrogen peroxide in the degradation of cytochrome P-450 by HpD photosensitization. As shown in Table 3, both superoxide dismutase and catalase did not protect against the destruction of microsomal cytochrome P-450, whereas scavengers of hydroxyl radicals such as benzoate, mannitol and ethyl alcohol (Anbar and Neta, 1967) afforded substantial protection against destruction of the cytochrome. Benzoate, mannitol and ethyl alcohol reduced the destruction of cytochrome P-450 caused by HpD and light 43, 24, and 24%, respectively.

Hematoporphyrin derivative absorbs radiant energy in the Soret band near 400 nm and is raised to an excited state. This energy of excited state of HpD can be transferred to the ground or triplet state of oxygen to generate singlet oxygen (Spikes and Straight, 1967; Nilsson and Kearns, 1973). Since scavengers of singlet oxygen such as 2,5-DMF, histidine, β-carotene, and ascorbic acid, substantially reduced the destruction of cytochrome P-450 by HpD and light, it is likely that singlet oxygen is involved in this damaging reaction. Scavangers of hydroxyl radicals such as benzoate, mannitol and

ethyl alcohol also offered protection against the destruction of cytochrome P-450. It is, therefore, possible that the hydroxyl radical is also generated during photosensitization by HpD and is associated with the degradation process. Kellogg and Fridovich (1975) suggested that scavengers of this moiety normally present in cells make it unlikely that the hydroxyl radical would escape scavenging and thus survive to attack critical cellular macromolecules. Our data support the concept that singlet oxygen is formed during the reaction of HpD and light (~400 nm) and is a major oxidant involved in the destruction of membrane-bound cytochrome P-450. Since reactive forms of oxygen such as singlet oxygen are generated as a consequence of the electronic excitation of oxygen in several biological processes (Kellogg and Fridovich, 1975; Kahn, 1970), it may result in the destruction of cytochrome P-450.

Activated oxygen species generated during porphyrin photosensitization are known to cause irreversible damage to cellular membranes including lysosomes, mitochondria, and red blood cells (Spikes and Straight, 1967; Nilsson and Kearns, 1973). Our findings clearly show that microsomal membrane-bound cytochrome P-450 is a potential target for damage by reactive oxygen species. Since cytochrome P-450 is the terminal oxidase for the biotransformation of potentially toxic foreign chemicals, such as drugs, and carcinogens as well as endogenous steriods, hormones, cholesterol, and bile acids, its destruction by singlet oxygen could be catastrophic to the cell and to essential biological functions which depend upon this haem-protein.

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