Journal of Photoscience, Vol. 4, No. 2, pp. 41–48

STRUCTURAL PERTURBATIONS INDUCED BY PHOTODYNAMIC ACTION OF PORPHYRIN AGGREGATES ON PLASMA MEMBRANE AND MICROSOMES OF GLIOBLASTOMA CELLS

RAJESH SREENIVASAN, PREETI G. JOSHI, and NANDA B. JOSHI*
Department of Biophysics, National Institute of Mental Health and Neurosciences
Bangalore -560029, India

(Received 18 *April* 1997; *accepted* 10 *June* 1997*)*

Abstract – The plasma membrane and microsomes, isolated from the cells treated with hematoporphyrin derivative (HpD) for 1 and 24 h, accumulated the aggregated porphyrin. The quantity of aggregated porphyrin was same in the plasma membrane and microsomes after isolating them from cells treated with HpD for 1 h whereas the microsomes accumulated higher quantity of aggregated porphyrin when cells were treated with HpD for 24 h. Photodynamic action of aggregated porphyrin on plasma membrane and microsomes was investigated using lipid specific fluorescent probes: 1,6-diphenyl-1,3,5-hexatrine (DPH) and 1-(4-trimethylammonium), 6-diphenyl-1,3,5-hexatrine(TMA-DPH). The time dependent anisotropy of these probes in the membranes was measured and the decay of anisotropy was analyzed using wobbling in cone model. Upon irradiation both the plasma membrane and the microsomes showed an increase in the limiting anisotropy and order parameter and a decrease in the cone angle of the lipid probes. The increase in the limiting anisotropy was pronounced in membranes isolated from the cells treated with HpD for 24 h. Photoinduced change in the limiting anisotropy was dependent on the duration of incubation of cells with HpD before isolating the membranes. In both the membranes, the membrane core was affected more as compared to the outer leaflet. In addition to the structural changes, a decrease in Na^+-K^+-ATPase and NADPH cyt c reductase activity was also observed upon irradiation of HpD treated cells. Inhibition in NADPH cyt c reductase was more when cells were treated with HpD for 24 h, however, Na^+-K^+-ATPase activity did not depend on the duration of the treatment of cells with HpD before irradiation. Our results suggest that the extent of photoinduced perturbations in the membranes varies as a function of duration of the treatment of cells with HpD and the membrane core is more susceptible to the photodynamic action of aggregated porphyrin.

INTRODUCTION

Photosensitizing properties of certain dyes are being exploited in photoinactivation of tumor cells, viruses and bacteria[1-6]. Hematoporphyrin derivative (HpD)† is one of the widely used photosensitizer. *In vitro* studies have shown that the plasma membrane, mitochondria, cytosolic and lysosomal enzymes are susceptible to the photosensitization[7-8]. Cellular membranes have been proposed to play an important role in photodynamic cellular damage[9]. Earlier studies on photosensitization of erythrocyte ghosts and cultured cells have shown protein cross linking, lipid peroxidation, derangement of cellular ion homeostasis, inhibition of amino acid transport and inactivation of enzymes[10-16]. Most of the previous studies on the photoinduced alterations in the membranes have been performed in different cell types and very little is known about the photoinduced structural changes in membranes in a single cell system as a function of duration of HpD treatment. Our earlier studies have shown that the photodynamic cellular damage depends greatly on the time of incubation of cells with HpD[6]. Incubation of cells with HpD for longer duration enhanced the photoinduced cell death. Though , the total amount of HpD taken up by the cells after shorter and longer duration of HpD treatment was similar, a change in the fluorescence intensity distribution of cell bound HpD as a function of incubation time was found. Previously, we have shown that the photosensitization perturbs the structural organization of the plasma membrane in intact U-87MG cells and isolated membranes when cells were treated with HpD for a fixed duration[16,17]. In the present paper we have compared the photoinduced structural changes in plasma membrane and microsomes, isolated from U-87MG cells treated with HpD for shorter and longer durations. Our results show that these membranes accumulate

* To whom correspondence should be addressed.

† *Abbreviations:*HpD: Hematoporphyrin derivative; PDT: Photodynamic therapy; EMEM: Eagle's minimum essential medium; PBS : Phosphate buffered saline; DPH: 1,6-diphenyl-1,3,5-hexatrine; TMA-DPH: 1-(4trimethylammonium), 6-diphenyl-1,3,5-hexatrine ; ATP : Adenosine-5'triphosphate; HEPES : N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; CTAB: Cetyl trimethyl ammonium bromide.

aggregated porphyrin and suggest that the extent of photoinduced perturbations to the plasma membrane and microsomes vary with the duration of incubation of cells with HpD.

MATERIALS AND METHODS

Materials. Hematoporphyrin dihydrochloride(Hp), adenosine-5' -triphosphate, cytochrome c, NADPH, nystatin, ouabain, cetyl trimethyl ammonomium bromide(CTAB), trypsin, sodium pyruvate, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES) and Tris were purchased from Sigma Chemical Company, St.Louis, MO (USA). 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammonium), 6-diphenyl-1,3,5-hexatriene(TMA-DPH) were acquired from Molecular Probes Inc, Eugene, OR (USA). Eagle's minimum essential medium (EMEM), phosphate buffered saline (PBS) and trypsin were procured from HiMedia, Bombay (India). Antibiotics were purchased from Hindustan Antibiotics, Pune (India). Foetal calf serum was procured from Northumbria Biological Ltd., Carmlington (UK) and bovine serum was prepared in the laboratory. Hematoporphyrin derivative (HpD) was prepared by the method of Gomer and Dougherty[18]. All other analytical grade chemicals were from Glaxo Laboratories, Bombay (India). Plastic tissue culture flasks were obtained from Nunclon, Denmark whereas Roux culture bottles were from Borosil, Bombay (India).

Cell culture. The human glioma (U-87MG) cell line was obtained from ATCC, Rockville (USA). The cells were grown at 37 °C in plastic tissue culture flasks (Nunc) and maintained in EMEM supplemented with 1m*M* sodium pyruvate, 5% bovine serum and 5% foetal calf serum, 2.2 g/L Hepes, 50000 units/L benzyl penicillin, 3500 units/L streptomycin and 2.2 mg/L nystatin. The cells were routinely subcultured after 96 h of growth. Cells were also grown in one litre Roux bottles when required in large quantity.

Dye treatment and irradiation. Required amounts of HpD was directly added to the growth medium and the cells in monolayer were incubated at 37 °C with the dye for a specific duration. The dye treated monolayer was first washed with PBS to remove the free HpD and treated twice with 0.25% trypsin containing 0.03% EDTA. The cells were resuspended in either PBS or 0.32 *M* sucrose solution buffered with 20 m*M* Tris (pH 7.4) as required for the experiment. Irradiation of membranes was performed by two 40 W cool day light fluorescent tubes (Phillips, India) covered with a perspex sheet. This source has its emission in the range from 400 to 700 nm. The fluence rate at the position of the samples was 1 W/m^2 as measured by the Kyoritsu Illuminometer model 5200 (Kyorisu Electrical Instruments, Japan).

Isolation of plasma membrane and microsomes. Isolation of plasma membrane and microsomes was carried out by the method of Cotman *et al.*, with modifications[19]. U-87MG cells, grown in monolayer, were treated with HpD and were harvested as described above. Cells were resuspended in cold sucrose solution(0.32 *M* sucrose, 20 m*M* Tris, pH 7.4). Cells were homogenized by mild sonication and complete homogenization of the cells was ensured by examining the material under microscope. Cell homogenate was centrifuged at 5500g for 20 min in a Sorvall Combi-Plus ultracentrifuge using a T-641 swinging bucket rotor. The nuclear pellet obtained in this step was discarded and the supernatant was centrifuged at 76,000g for 25 min. The pellet obtained in this step consisted of plasma membrane and mitochondrial fractions whereas the supernatant consisted of microsomal fraction. The pellet was resuspended in sucrose solution (0.32 *M* sucrose, 20 m*M* Tris, pH 7.4) and layered over a high density sucrose solution (1.2 *M*). The ratio of the cell homogenate and high density sucrose solution (1.2 *M*) was 2:1. This gradient was centrifuged at 150,000g for 30 min. The resultant pellet was mitochondria while the interphase consisted of plasma membrane. The interphase was carefully removed and layered on a sucrose solution (0.8 *M*). This gradient was centrifuged at 150,000g for 30 min and the resulting pellet was plasma membrane. The supernatant consisting of microsomal fraction was also centrifuged at 150,000g for 30 min. The purity of these preparations was assessed by measuring the activity of marker enzymes in each fraction in comparison to the activity of the enzyme in the homogenate. The purity of each preparation was found to be 60%.

Enzyme assays. The activity of the Na^+-K^+-ATPase was measured by the method of Deliconstantinos *et al.*[20]. The assay was performed in two sets of tubes, containing 50 m*M* Tris (pH 7.3), 80 m*M* NaCl, 20 m*M* KCl 3 m*M* $MgCl_2$, membrane sample (150 μg protein) and 3 m*M* adenosine-5'-triphosphate in a total reaction volume of 0.5 mL with and without 0.5 m*M* ouabain. The reaction was initiated by the addition of ATP and was stopped after 30 min with the addition of 10% ice cold TCA.

The activity of the enzyme NADPH cyt c reductase was measured by the method of Guengerich[21]. The reaction mixture contained 30 m*M* PB 0.16 m*M* KCl, 0.04% cytochrome c, membrane sample (300 μg protein) and 0.8 m*M* NADPH, in a total volume of 1.2 mL. The reaction was initiated by the addition of NADPH and the optical density was monitored at 550 nm. The specific activity of the enzyme was calculated using extinction coefficient (ϵ) 2.77×10^4 $M^{-1}cm^{-1}$ for cytochrome c at 550 nm.

HpD uptake measurements. The uptake of HpD by the cells and membranes was measured by the method of Christensen *et al*[22]. The required amount of cells or membrane fractions was dissolved in 1% cetyl trimethyl ammonium bromide (CTAB) which was prepared in 0.2 N NaOH. The optical density was measured using a JASCO UV-VIS spectrophotometer. HpD content in the cells or membrane fractions were estimated by a standard curve obtained by dissolving known quantities of HpD in 1% CTAB with 0.2 N NaOH. The HpD content was calculated per unit protein. For the membrane fractions the HpD content in each fraction was expressed as a percentage of the total HpD taken up by the cells.

Labeling of membranes with fluorescent probes. Membranes were labeled with DPH and TMA-DPH by the methods of Shinitzky and Inbar[23] and Petty *et al.*[24], respectively. Briefly,

the stock solution (5 m*M*) of DPH in THF was diluted 250 fold by adding the same to PBS and the solution was stirred continuously for 1 h. A known amount of this solution was mixed with an equal volume of membrane suspension (200 μg/ml protein) and incubated at 25 °C for 30 min. The incorporation of DPH into the membranes was followed by a steep increase in the fluorescence intensity of DPH. The procedure for labeling the membranes with TMA-DPH was similar to that of DPH but the incubation time was only 10 min.

Measurement of fluorescence spectra. The fluorescence spectra of HpD bound to cells or membrane fractions were recorded on a SLM 8000C spectrofluorometer. Samples were excited at 395 nm and the emitted photons were selected by an emission monochromator and detected by a cooled photomultiplier tube.

Measurement of decay time and time dependent anisotropy. The decay of fluorescence intensity and anisotropy was measured using a Edinburgh CD900 time resolved spectrofluorometer, which utilizes the single photon counting method to generate the decay curves. The light source was a N_2 discharge lamp operated at a pressure of 1 bar. The excitation wavelength was 337 nm and the emission was monitored at 415 nm for both DPH and TMA-DPH. The data were collected in 1024 channels using a multichannel analyzer. In each experiment the data were acquired to give a peak counts of about 5000. For each sample decay curve, a corresponding lamp profile using a scattering solution was collected. Data were analyzed by nonlinear least square deconvolution procedure[25]. The goodness of fit was tested by chi-square and residuals.

The decay of fluorescence anisotropy was measured by incorporating the Glan Thompson calcite prism polarizers in the excitation and emission paths. Samples were excited with vertically polarized light. The decay of vertically [$I_{vv}(t)$] and horizontally [$I_{vh}(t)$]polarized fluorescence intensities were measured by collecting the data alternately between two memory segments of multichannel analyzer. The toggling of the emission polarizer between vertical and horizontal positions with simultaneous change in memory segment was automatically controlled with a dwell time of 30 sec. The decay of anisotropy was generated from the measured time resolved decay of $I_{vv}(t)$ and $I_{vh}(t)$ using the equation:

$$r(t) = I_{vv}(t) - G\, I_{vh}(t) \,/\, I_{vv}(t) + 2\, G\, I_{vh}(t) \quad (1)$$

where G is the correction factor given by $G = I_{hv}(t)/I_{hh}(t)$. $I_{hv}(t)$ and $I_{hh}(t)$ are the vertically and horizontally polarized fluorescence intensities on exciting the sample using horizontally polarized light. The decay of anisotropy of fluorophore in the membrane has been explained in terms of the wobbling in cone model[26-28]. According to this model, the fluorophore is considered to be in a hindered environment and its orientational motion in the membrane is described by the wobbling confined within a cone around the normal to the membrane. The decay of fluorescence anisotropy of a fluorophore in the hindered environment like membrane is represented by:

$$r(t) = (r_0 - r_\infty)\, e^{-t/\Phi} + r_\infty \quad (2)$$

where r_0 is the anisotropy at t = 0, r_∞ is the limiting anisotropy and Φ is the rotational relaxation time for the wobbling diffusion within the cone. The relationship between the limiting anisotropy, order parameter and the cone angle is given by

$$r_\infty/r_0 = S^2 = (1/4)\,[\cos\theta_c\,(1 + \cos\theta_c)]^2 \quad (3)$$

where S is order parameter and θ_c is cone angle.

RESULTS

HpD binding to plasma membranes and microsomes

To understand the photoinduced alterations in the plasma membrane and microsomes it was necessary to study the binding of HpD to these membranes. Figure 1A shows the emission spectra of HpD bound to the plasma membrane and the microsomes isolated from the cells treated with HpD for 1 h. The emission spectrum of HpD bound to the intact cells has also been shown for comparison. It is clear that the emission spectra of HpD bound to the membranes are different from that of cell bound HpD. The emission spectrum of HpD bound to

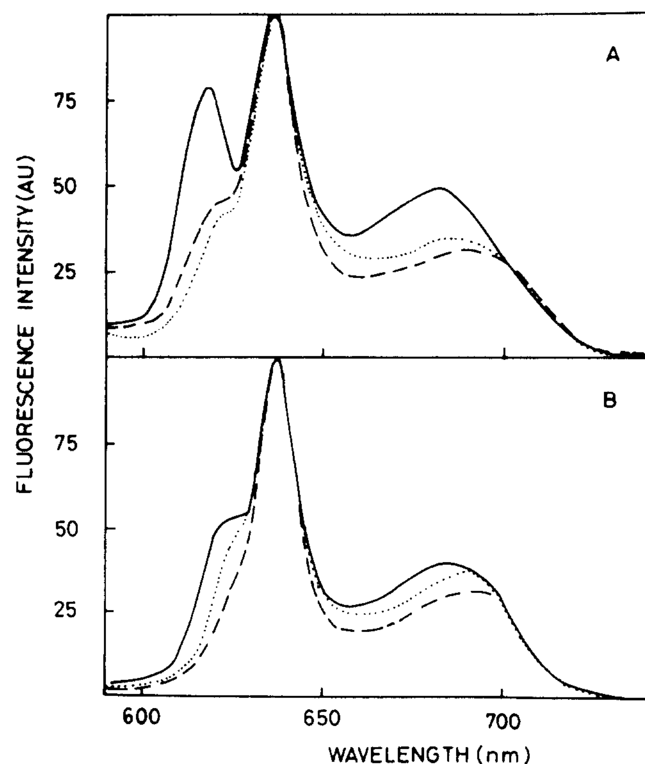

Figure 1. Fluorescence spectra of dye bound to (- - - -) plasma membrane, (⋯⋯) microsomes and (——) U-87MG cells. Plasma membrane and microsomes were isolated as described in the method section. Membranes were isolated after treating the cells with HpD for (A) 1 h and (B) 24 h.

Table 1. Fluorescence decay times and fractional contributions of TMA-DPH incorporated into the membranes isolated from the cells treated with HpD for 1 and 24 h. τ_1 and τ_2 are the fluorescence decay times whereas f_1 and f_2 are the corresponding fractional contributions. The numbers shown in the parenthesis are the fractional contributions.

Sample		Decay time(ns)/Fractional τ_1/f_1	contribution τ_2/f_2	Chi-square χ^2
Plasma Membrane	1 h	1.80 ± 0.07 (0.31)	5.45 ± 0.05 (0.69)	1.10
	24 h	1.93 ± 0.05 (0.41)	6.61 ± 0.06 (0.59)	1.73
Microsomes	1 h	2.35 ± 0.09 (0.33)	5.97 ± 0.75 (0.67)	1.19
	24 h	1.58 ± 0.04 (0.45)	5.16 ± 0.06 (0.55)	1.10

Table 2. Fluorescence decay times and fractional contributions of DPH incorporated into the membranes isolated from the cells treated with HpD for 1 and 24 h. τ_1 and τ_2 are the fluorescence decay times whereas f_1 and f_2 are corresponding fractional contributions. The numbers shown in the parenthesis are the fractional contributions.

Sample		Decay time(ns)/Fractional τ_1/f_1	contribution τ_2/f_2	Chi-square χ^2
Plasma Membrane	1 h	2.73 ± 0.41 (0.28)	8.97 ± 0.04 (0.72)	1.28
	24 h	3.26 ± 0.06 (0.45)	9.01 ± 0.10 (0.55)	1.10
Microsomes	1 h	2.17 ± 0.07 (0.22)	8.23 ± 0.05 (0.78)	1.10
	24 h	2.01 ± 0.05 (0.32)	7.36 ± 0.05 (0.68)	1.10

the cells exhibits three fluorescence bands at 615, 636 and 676 nm while the emission spectrum of HpD bound to either of the membranes exhibited an emission maximum at 636 nm with a shoulder at 626 nm and a broad band at 696 nm. Similar measurements were performed in the membranes isolated from cells treated with HpD for 24 h and the results are shown in Fig. 1B.

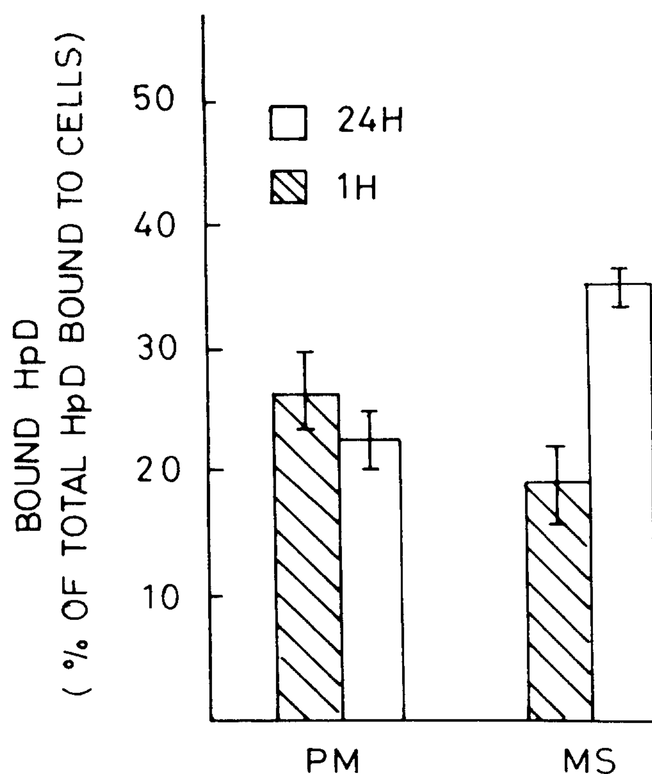

Figure 2. Comparison of HpD taken up by plasma membrane (PM) and microsomes (MS) isolated from cells treated with HpD for 1 h and 24 h. The values given are the percentage of total HpD taken up cells.

HpD uptake by membranes

A comparison of the HpD uptake by plasma membrane and microsomes, isolated from cells treated with HpD for 1 h as well as 24 h, is depicted in Fig. 2. The total HpD taken by the cells was normalized to 100% and the accumulation of HpD by the membranes was determined with respect to the total HpD accumulated by the cells. We observed that the plasma membrane accumulates more HpD than microsomal membranes when cells were treated with HpD for 1 h. In cells treated with HpD for 24 h, the quantity of HpD accumulated in microsomal fraction was significantly higher as compared to the plasma membrane fraction.

Decay time of the lipid probes

The decay of fluorescence intensity of TMA-DPH and DPH incorporated into the isolated membranes showed a double exponential decay. The fluorescence life times along with the fractional contributions are given in Table 1 and 2. No significant changes were observed in the decay times of either DPH or TMA-DPH in any of the membranes as a result of photosensitization.

Time dependent anisotropy of TMA-DPH

The decay of anisotropy, analyzed using Eq. (2), exhibited the best fit for a single rotational correlation time. The limiting anisotropy (r_∞) and rotational correlation time (Φ) for TMA-DPH incorporated in the membranes are given in Table 3. HpD treatment alone for 1 h did not cause any appreciable change in the limiting anisotropy or rotational correlation time. The irradiation of the plasma membrane and microsomal fractions, isolated from cells treated with HpD for 1 h, caused no significant change in the limiting anisotropy and rotational correlational time of TMA-DPH.

Table 3. Limiting anisotropy (r_∞) and rotational correlation time (Φ) of TMA-DPH incorporated into the plasma membrane and microsomes with and without irradiation. Plasma membrane and microsomes were isolated from U-87MG cells treated with HpD for 1 h and 24 h.

Sample		Dark controls		Irradiated	
		r_∞	Φ(ns)	r_∞	Φ(ns)
Plasma Membrane	1 h	0.219 ± 0.003	2.46 ± 0.32	$0.224 \pm 0.003^*$	2.58 ± 0.39
	24 h	0.226 ± 0.004	2.95 ± 1.14	$0.238 \pm 0.005^{**}$	3.70 ± 0.34
Microsomes	1 h	0.220 ± 0.003	3.16 ± 0.37	$0.233 \pm 0.008^*$	3.10 ± 0.93
	24 h	0.216 ± 0.007	3.85 ± 0.14	$0.247 \pm 0.006^{***}$	3.65 ± 1.27

* Not significant
** Significantly different at $p < 0.05$ than dark controls
*** Significantly different at $p < 0.005$ than dark controls

Irradiation of the membrane fractions, isolated from cells treated with HpD for 24 h caused an increase in the limiting anisotropy of TMA-DPH by 5.3% ($p < 0.05$) in the plasma membrane and by 14.3% ($p < 0.01$) in the microsomes. However, the rotational correlation time did not change significantly.

Time dependent anisotropy of DPH

The values of limiting anisotropy (r_∞) and rotational correlation time (Φ) of DPH incorporated into the membranes are shown in table 4. Irradiation of the plasma membrane isolated from the cells treated with HpD for 1 h increased the limiting anisotropy of DPH by 14.9% ($p < 0.05$). However, there was no significant change in the r_∞ of DPH incorporated into the microsomal membrane. Irradiation of the plasma membrane and microsomes isolated from cells treated with HpD for 24 h increased the limiting anisotropy by 26.2% ($p < 0.025$) and 18.5% ($p < 0.025$) respectively. There was no significant change in the rotational correlation time in either of the membranes upon irradiation.

Table 4. Limiting anisotropy (r_∞) and rotational correlation time (Φ) of DPH incorporated into the plasma membrane and microsomes with and without irradiation. Plasma membrane and microsomes were isolated from U-87MG cells treated with HpD for 1 h and 24 h.

Sample		Dark controls		Irradiated	
		r_∞	Φ(ns)	r_∞	Φ(ns)
Plasma Membrane	1 h	0.121 ± 0.003	3.44 ± 1.03	$0.139 \pm 0.007^+$	2.73 ± 0.09
	24 h	0.126 ± 0.011	3.83 ± 1.05	$0.159 \pm 0.002^{++}$	4.90 ± 0.17
Microsomes	1 h	0.149 ± 0.001	4.15 ± 1.28	$0.160 \pm 0.007^{**}$	3.36 ± 0.45
	24 h	0.130 ± 0.001	5.54 ± 0.55	$0.154 \pm 0.008^{***}$	5.11 ± 0.55

+Significantly different at $p < 0.025$ than dark controls
++Significantly different at $p < 0.01$ than dark controls
** Not Significant
*** Significantly different at $p < 0.005$ than dark controls

Estimation of order parameter and cone angle

The order parameter (S) and cone angle (θ_c) were calculated from the limiting anisotropy values using Eq.

Table 5. Order parameter (S) and cone angle (θ_c) of TMA-DPH incorporated into the plasma membrane and microsomes with and without irradiation, Plasma membrane and microsomes were isolated from U-87MG cells treated with HpD for 1 h and 24 h.

Sample		Dark controls		Irradiated	
		S	θ_c	S	θ_c
Plasma Membrane	1 h	0.749 ± 0.005	34.7	$0.757 \pm 0.005^*$	34.0
	24 h	0.761 ± 0.007	33.8	$0.781 \pm 0.008^{**}$	32.2
Microsomes	1 h	0.751 ± 0.005	34.5	$0.772 \pm 0.013^*$	32.8
	24 h	0.744 ± 0.012	35.1	$0.795 \pm 0.019^{***}$	31.0

* Not significant
** Significantly different at $p < 0.05$ than dark controls
*** Significantly different at $p < 0.005$ than dark controls

Table 6. Order parameter (S) and cone angle(θ_c of DPH incorporated into the plasma membrane and microsomes with and without irradiation. Plasma membrane and microsomes were isolated from U-87MG cells treated with HpD for 1 h and 24 h.

Sample		Dark controls		Irradiated	
		S	θ_c	S	θ_c
Plasma Membrane	1 h	0.557 ± 0.009	48.7	$0.597 \pm 0.015^+$	45.4
	24 h	0.568 ± 0.025	47.3	$0.638 \pm 0.004^{++}$	42.6
Microsomes	1 h	0.618 ± 0.002	44.0	$0.640 \pm 0.014^{**}$	42.5
	24 h	0.577 ± 0.002	46.7	$0.628 \pm 0.016^{***}$	43.3

+ Significantly different at $p < 0.025$ than dark controls
++ Significantly different at $p < 0.01$ than dark controls
** Not Significant
**** Significantly different at $p < 0.005$ than dark controls

Table 7 . Activity of Na^+-K^+-ATPase (nmoles Pi/min/mg) and NADPH cyt c reductase (μmoles P_i/min/mg) in cells treated with HpD for 1 and 24 h with and without irradiation.

Enzyme		Activity	
		Dark controls	Irradiated
Na^+-K^+-ATPase	1 h	65.30 ± 12.30	11.94 ± 7.30
	24 h	66.90 ± 15.30	11.50 ± 7.45
NADPH cyt c reductase	1 h	1.99 ± 0.03	0.72 ± 0.13
	24 h	2.02 ± 0.06	0.23 ± 0.07

(3). The plasma membrane and microsomes, isolated from cells treated with HpD for 1 h, showed no significant change either in the order parameter or in the cone angle of TMA-DPH. However, in the membranes isolated from cells treated with HpD for 24 h, the order parameter increased while the cone angle decreased in both the membranes, as shown in Table 5.

The order parameter and cone angle for DPH incorporated into the membranes differed from the values for TMA-DPH. In the membranes isolated from cells treated with HpD for 1 h, the order parameter increased and the cone angle decreased for DPH in the plasma membrane, while the microsomal membrane showed no significant change. However, in the membranes isolated from cells treated with HpD for 24 h the order parameter of DPH increased in the plasma membrane and microsomal membranes, while the cone angle decreased in both the membranes. These results are shown in Table 6.

Na^+-K^+-ATPase and NADPH cyt c reductase activity

Table 7 depicts the activity of Na^+-K^+-ATPase and NADPH cyt c reductase in cells treated with HpD for 1 h and 24 h with and without irradiation. The activity of the enzymes in cells treated with HpD alone for 1 or 24 h was similar to the untreated controls. The activity of Na^+-K^+-ATPase was found to be 65.3 ± 12.3 nmoles Pi/min/mg protein in cells treated with HpD for 1 h. Irradiation to the cells reduced the enzyme activity to 11.9 ± 7.3 nmoles Pi/min/mg protein. In cells treated with HpD for 24 h the activity of Na^+-K^+-ATPase was inhibited to the same extent after irradiation as in the cells treated with HpD for 1 h. The activity of NADPH cyt c reductase was found to be 1.99 ± 0.30 μmoles/min/mg protein in cells treated with HpD for 1 h which was inhibited to 0.72 ± 0.13 μmoles/min/mg protein after irradiation. In cells treated with HpD for 24 h, NADPH cyt c reductase activity was 2.02 ± 0.06 μmoles/min/mg protein and on irradiation the activity reduced to 0.230 ± 0.07 μmoles/min/mg protein.

DISCUSSION

Our earlier studies on HpD binding to intact cells have shown that the fluorescence intensity distribution of cell bound HpD was different in cells treated with HpD for different durations[6,29]. It has been suggested that the emission band at 636 nm in the emission spectra of HpD bound to the cells originates due to the binding of aggregated porphyrin to the hydrophobic sites in the cells whereas the emission at 615 nm was due to the binding of monomeric HpD to the hydrophilic sites in the cells. The present study demonstrate a typical fluorescence intensity distribution of HpD bound to the membranes, irrespective of whether they were isolated from cells treated with HpD for 1 h or 24h. HpD bound to the isolated membranes exhibited an emission maximum at 636 nm with a shoulder at 620 nm and a broad band at 696 nm. The absence of 615 nm band in the spectra of membrane bound HpD clearly suggest that these membranes accumulate the aggregated porphyrin. The reason for the absence of 615 nm band in the spectra of membrane bound HpD is not known at present. The measurement of the HpD accumulation by the membranes showed that after shorter duration of HpD treatment the plasma membrane accumulated higher quantity of dye as compared to the microsomal fraction. However, after longer duration of HpD treatment, the accumulation of HpD was significantly higher in microsomal fraction than in the plasma membrane fraction, though, the quantity of HpD taken up by the plasma membrane was similar after 1 or 24 h of incubation. Our results are contrary to the earlier suggestions that HpD localizes only in the plasma membrane after shorter duration of incubation and it migrates from the plasma membrane to intracellular sites on increasing the duration of incubation.[8] Our results showed that microsomes accumulate HpD even after shorter duration of incubation. The increase in HpD accumulation by microsomes on longer duration of incubation may be due to the diffusion of HpD from extracellular medium instead of the migration of HpD from plasma membrane to the microsomes as suggested earlier.

Our results on HpD binding demonstrated that both plasma membrane and microsomes accumulate the aggregated porphyrin. Therefore, these membranes are most suitable systems to investigate the photoinduced changes caused by aggregated porphyrin which has been shown to be a tumor localizing and photosensitizing component present in HpD. Our results showed that the anisotropy of TMA-DPH or DPH incorporated into the membranes isolated from cells treated with HpD alone for either 1 h or 24 h was similar to that observed in untreated controls. On irradiation, an increase in the anisotropy of the lipid probes incorporated into the membranes was found, but this increase was dependent on the duration of treatment of cells with the dye before isolating the membranes, the nature of the lipid probes

and also on the membrane fractions. In both the membranes, the anisotropy of DPH showed a large increase as compared to TMA-DPH. DPH is a rod shaped hydrophobic molecule and partitions into the lipid core of the biological membranes while TMA-DPH is a cationic derivative of DPH and partitions in the bilayer leaflet[30]. These two probes can give the structural information on the different portions in the membranes. Our results suggest that the effect of photosensitization is larger on the lipid core of the membrane as compared to the outer leaflet. It is possible that the aggregated porphyrin localizes in the core of the membrane lipid bilayer. The accumulation of aggregated porphyrin in the membrane core could be more with the increase in the duration of the HpD treatment to the cells which may be responsible for the larger changes in the anisotropy of DPH in the membranes isolated from the cells treated with HpD for 24 h. The more photosensitivity of the plasma membrane as compared to the microsomes may be due to the variations in the lipid-protein ratio and the lipid composition of these membranes.

The increase in the limiting anisotropy of the probes implies a more hindered motion of the probe molecule in the membrane. The limiting anisotropy of TMA-DPH in plasma membrane of intact U-87MG cells treated with HpD for lh was found to be 0.250 ± 0.002[16] whereas the anisotropy of TMA-DPH in isolated plasma membrane obtained in the present study is 0.219 ± 0.003 These results suggest that the isolated plasma membrane is more disordered than that in the intact cells. The time resolved anisotropy measurements can provide information on both the rate and range of the rotational motions of the probe molecules which give the dynamic and static information about the mobility of the probes in the membranes[26-28]. The increase in the order parameter and a decrease in the cone angle suggest that the photosensitization affects the range of the rotational motion of the probes. On the other hand, the rate of the rotational motion is not affected by photosensitization as evidenced by no change in the rotational relaxation time.

The increase in the membrane order on photosensitization may arise due to the cross linking of lipid radicals and/or as a consequence of the formation of lipid peroxides[31,32]. Earlier studies have shown that HpD mediated photosensitization can induce lipid peroxidation in the plasma membrane and microsomes[12,16]. Lipid hydroperoxides are formed by free radical attack at unsaturated fatty acyl chains. Such hydroperoxides can then decompose with the liberation of malondialdehyde. The removal of unsaturation from fatty acyl chains allow the saturated hydrocarbon chains to pack more closely and make the membrane more ordered.

Structural changes in the membranes as a result of photosensitization lead to the changes in the functional parameters. We observed that HpD induced photosensitization inhibits the activity of plasma membrane enzyme Na^+-K^+-ATPase but the inhibition does not depend on the duration of HpD treatment. Our results are in concurrence with those of Gibson *et al*[11]. They have shown that Na^+-K^+-ATPase is highly susceptible to photosensitization even after very short duration of HpD treatment. In contrast to Na^+-K^+-ATPase, the photoinduced changes in the activity of NADPH cyt c reductase was found to be dependent on the duration of HpD treatment. In cells treated with HpD for 1 and 24 h, NADPH cyt c reductase activity was inhibited to 35% and 10% respectively of its value in the dark control. These results suggest that the photosensitization of NADPH cyt c reductase is highly susceptible to the duration of HpD treatment. However, the fact that the HpD accumulation by the microsomal membrane was higher than the plasma membrane can not be neglected. It is speculated that the microsomal membrane offers specific binding sites for the oligomeric component of HpD. In summary, both plasma membrane and microsomes accumulate aggregated porphyrin. The photoinduced structural alterations observed in the plasma membrane and microsomes suggest that the plasma membrane is more susceptible to photosensitization than the microsomal membrane. These results also suggest that the membrane core is affected more as compared to the outer leaflet.

Acknowledgements – This work was supported by Department of Science and Technology, Govt. of India.

REFERENCES

1. Kaye, A. H., G. Morstyn and M. L. J. Apuzzo (1988) Photoradiation therapy and its potential in the management of neurological tumors. *J. Neurosurg.* **69**, 1-14.
2. Dougherty, T. J. (1993) Photoradiation therapy. *Photochem. Photobiol.* **58**, 895-900.
3. Moan, J. and K. Berg (1992) Photochemotherapy of cancerexperimental research. *Photochem. Photobiol.* **55**, 931-948.
4. Spikes, J. D. and G. Jori (1987) Photodynamic therapy of tumors and other diseases using porphyrins. *Lasers Med. Sci.* **2/3**, 3-15.
5. Sieber, F., J. M. O'Brien, G. J. Krueger, S. L. Schober, W. H. Burns, S. J. Sharkis and L. L. Sensenbrenner (1987) Antiviral activity of merocyanine 540. *Photochem. Photobiol.* **46**, 707-711.
6. Joshi, K., P. G. Joshi and N. B. Joshi (1992) Binding of hematoporphyrin derivative to brain tumor cells- a fluorescence spectroscopic study. *Photochem. Photobiol.* **56**, 113-118.
7. Jori, G., and J. D. Spikes (1984) Photobiochemistry of porphyrins. In *Topics in Photomedicine* (Edited by K. C. Smith) pp. 183-318, *Plenum Press, New. York.*

8. Kessel, D. (1986) Sites of photosensitization by derivative of hematoporphyrin. *Photochem. Photobiol.* **46**, 489-493.
9. Valenzeno, D. P. (1987) Photomodification of biological membranes with emphasis on singlet oxygen mechanism. *Photochem. Photobiol.* **46**, 147-160.
10. Dubbelman, T. M. A. R., A. F. P. M. DeGoeij, K. Christiansen and J. Van Stevninck (1981) Protoporphyrin induced photodynamic effect on band-3 protein of human erythrocyte membranes. *Biochem. Biophys. Acta* **649**, 310-316.
11. Gibson, S. L., R. S. Murant and R. Hilf (1988) Photosensitizing effect of hematoporphyrin derivative and photofrin II on plasma membrane enzymes 5'-nucleotide, Na^{+}-K^{+}-ATPase and Mg^{2+}-ATPase in R3230 AC mammary adenocarcinoma. *Cancer Res.* **48**, 3360-3366.
12. Athar, M., H. Muktar and D. R. Bickers (1988) Differential role of reactive oxygen intermediates in photofrin I and photofrin II mediated photoenhancement of lipid peroxidation in epidermal microsomal membrane. *J. Invest. Dermatol.* **90**, 652-657.
13. Specht, K. G. and M. A. J. Rodgers (1991) Plasma membrane depolarization and calcium influx during cell injury by photodynamic action. *Biochem. Biophys. Acta* **1070**, 60-68.
14. Joshi, P. G., K. Joshi, S. Mishra and N. B. Joshi (1994) Ca^{2+} influx induced by photodynamic action in human cerebral glioma cells: possible involvement of a calcium channel. *Photochem. Photobiol.* **60**, 244-248.
15. Dellinger, M., F. Ricchelli, G. Moreno and C. Salet (1994) Hematoporphyrin derivative (photofrin) photodynamic action on Ca^{2+} transport in monkey kidney cells (CV-1). *Photochem. Photobiol.* **60**, 368-372.
16. Joshi, K., P. G. Joshi and N. B. Joshi (1995) Structural alterations induced by photodynamic action of hematoporphyrin derivative in plasma membrane of glioblastoma cells: time dependent fluorescence spectroscopic study. *Ind. J. Biochem. Biophys.* **32**, 200-206.
17. Sreenivasan, R., P. G. Joshi and N. B. Joshi (1997) Hematoporphyrin derivative induced photodamage to brain tumor cells: alteration in subcellular membranes. *Rad. Phys. Chem.* **49**, 145-149.
18. Gomer, C. and T. J. Dougherty (1979) Determination of ^{3}H and ^{14}C HpD distribution in malignant and normal tissue. *Cancer Res.* **39**, 146-151.
19. Cotman, C., H. Herschman and D. Taylor (1971) Subcellular fractionation of cultured glial cells. *J. Neurobiol.* **2**, 169-180.
20. Deliconstantinos, G., K. T. Luidmila and V. Vassilik (1987) Evaluation of membrane fluidity effects and enzyme activity alterations in adriamycin neurotoxicity. *Biochem. Pharma.* **36**, 1153-1161.
21. Guengerich, P. P. (1984) Microsomal enzymes involved in toxicity-analysis and separation. In *Principles and Methods of Toxicology.* (Edited by A. W. Hayes) pp. 609-634. Raven Press, New York.
22. Christensen, T., T. Sandquist, K. Feven, H. Wakshvik and J. Moan (1983) Retention and photodynamic effects of hematoporphyrin derivative in cells after prolonged cultivation in the presence of porphyrins. *Br. J. Cancer* **48**, 35-43.
23. Shintzsky, M. and M. Inbar (1976) Microviscosity parameters and protein mobility in biological membranes. *Biochem. Biophys. Acta* **433**, 133-149.
24. Petty, H. R., C. D. Neibylski and J. W. Francis (1987) Influence of immune complexes on macrophase membrane fluidity: a nanosecond fluorescence anisotropy study. *Biochemistry* **26**, 6340-6348.
25. Lakowicz, J. R. (1983) In *Principles of Fluorescence Spectroscopy.* Plenum Press, New York.
26. Kinosita, K., S. Kawato and A. Ikegami (1977) A theory of fluorescence polarization decay in membranes. *Biophys. J.* **20**, 289-305.
27. Hildenbrand, K. and C. Nicolan (1979) Nanosecond fluorescence anisotropy decays of 1,6-diphenyl-1,3,5-hexatrine in membranes. *Biochem. Biophys. Acta* **553**, 365-377.
28. Kinosita, K., K. Kataoka, Y. Kimura, O. Gotoh and R. Ekegami (1981) Dynamic structure of biological membranes as probed by 1,6-diphenyl-1,3,5-hexatrine: a nanosecond fluorescence depolarization study. *Biochemistry* **20**, 4270-4277.
29. Sreenivasan, R., P. G. Joshi and N. B. Joshi (1995) Hematoporphyrin derivative binding and photosensitization in human glioblastoma cells: comparison of exponential and plateau phase cells. *Ind. J. Expt. Biol.* **32**, 763-766.
30. Prendergast, G., R. Haugland and P. J. Callahan (1981) 1-(4-Trimethylammonium), 6-diphenyl-1,3,5-hexatrine synthesis, fluorescence properties and use as a fluorescent probes of lipid bilayers. *Biochemistry* **20**, 7333- 7338.
31. Barber, D. J. W. and J. K. Thomas (1978) Reactions of radicals with lecithin bilayers. *Rad. Res.* **75**, 51-65.
32. Dobretsov, G. E., T. A. Borscheievskaya, V. A. Petrov and Y. A. Vladimirov (1974) The increase of phospholipid bilayer rigidity after lipid peroxidation. *FEBS Letts.* **84**, 125-128.