PHOTODAMAGE TO HEPATOCYTES BY VISIBLE LIGHT

Lydia Y. L. CHENG and Lester PACKER

Membrane Bioenergetics Group, Lawrence Berkeley Laboratory and *the Department of Physiology-Anatomy, University of California, Berkeley, CA 94720 USA*

Received 24 October 1978

1. Introduction

There have been several recent reports of the damaging effects of visible light exposure (>400 nm) on various microorganisms [1,2] and cultured mammalian cells [3–5]. WI-38 human fibroblasts show a gradual decline in growth rate with exposure to visible light [6] with younger cells being more susceptible to photokilling and with partial protection observed by DL-α-tocopherol or vitamin E. Visible light-absorbing chromophores and vitamin E are largely associated with the inner mitochondrial membrane. A previous study made on the effect of visible light exposure to isolated mitochondria has indicated a flavin-photosensitized initiation process and oxygen-dependent damage to energy coupling, flavin dehydrogenases and quinones, but not to hemoproteins of the inner membrane. In this study, with isolated hepatocytes, the pattern of intracellular damage was studied with a view towards clarifying the mechanism of cellular photokilling.

Exposure of rat hepatocytes to visible light (400–720 nm) of intensity 300 mW/cm^2 results in a selective pattern of subcellular damage. Plasma membrane permeability and enzymes are largely unaffected, whereas inactivation occurs to inner mitochondrial membrane enzymes in vivo in a pattern similar to exposure of mitochondria in vitro. The most sensitive parameters of damage were inactivation of catalase and inactivation of lysosomal enzymes. Partial protection to lipid against visible light damage is afforded by membrane antioxidants like DL-α-tocopherol. The same effects are also observed in hepatocytes from animals grown on diets deficient and supplemented with DL-α-tocopherol. Partial protection to proteins against visible light damage is afforded by reducing conditions, substrate + KCN and proton-donating reagents. The results indicate that both flavins and hemes may serve as the visible light-absorbing chromophores (>400 nm) to propagate intracellular damage which results in photokilling of mammalian cells.

2. Methods

Hepatocytes were isolated from 280–300 g male Long-Evans rats according to [7]. Briefly, this involves liver perfusion with Ca^{2+}-free buffer followed by perfusion with collagenase. The dispersed hepatocytes were washed 3 times in ice cold 0.25 M sucrose by centrifugation and resuspended in 10 vol. 0.25 M sucrose. For experiments they were illuminated with 300 mW/cm^2 visible light 400–720 nm as in [8] together with dark controls.

2.1. *Assays*

Enzyme assays were as follows: cytochrome *c* oxidase, catalase and *N*-acetyl-β-glucosaminidase [9]; succinate dehydrogenase and succinate oxidase [8]; glucose-6-phosphatase and 5′-nucleotidase as in [10] except that half the substrate concentration was employed. Phosphate was determined as in [11]. Lipid peroxidation by the method in [12] as described in [8]. NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase were measured in the presence of 1 mM KCN as in [13]. Several other assays were modified.

Glutathione peroxidase was adapted from [14] as follows. a reaction mixture (20 ml) 50 mM Tris–chloride (pH 7.6), 0.1 mM EDTA, 0.12 mM NADH

and 0.28 M glutathione were mixed with 12 units of a glutathione reductase solution (Sigma Chemical Company). The reaction was initiated by adding 0.1 ml sonicated cell suspension to 0.9 ml of the above mixture. At equilibrium the reaction was initiated by addition of 30 μl of 6.6 mM solution of cumene hydroperoxide (Polysciences Inc.).

Urate oxidase was assayed by a modification of [15] as follows A reaction mixture (0.9 ml) of 30 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA and 0.1% Triton X100 (w/w) was mixed with 0.1 ml sonicated cell suspension, equilibrated for 3–5 min: the reaction was commenced by addition to 20 μl 4.2 mM sodium urate. A molar extinction coefficient of $E_{(292\ nm)}$ = 12 200 was employed for calculation of its disappearance [16].

For measurement of total enzyme activity hepatocytes were sonicated for 2 min at 4°C with 30 s intervals in a Branson sonifier model 350. Latency was estimated as in [9], viz. cells were homogenized by 10 up-and-down strokes in a Dounce Type B homogenizer, centrifuged at 50 × *g* to remove unbroken cells and activity assayed in the supernatant fraction.

Cell viability was determined by the trypan blue exclusion technique [17] and by the extent of release of lactate dehydrogenase activity.

Butylated hydroxytoluene and DL-α-tocopherol were dissolved in absolute ethanol to give 1 mM or 100 μM solutions. They were then added to the hepatocyte suspension to the final concentrations indicated in fig.2.

Vitamin E-deficient and supplemented rats: Sprague-Dawley male rats, 120 g, were fed at least 4 weeks on a DL-α-tocopherol supplemented (Bio-Mix no. 799) or deficient (Bio-Mix no. 810) diet (Hoffman-La Roche, Inc., Nutley, NJ) before being used. Hepatocytes were prepared from both groups on the same day and adjusted to the same protein concentration before illuminated. Assays of lipid peroxidation and enzymes were as in [8].

3. Results

Over a 12 h period of light exposure no release of lactate dehydrogenase or uptake of trypan blue was observed (table 1). Also plasma membrane enzymes 5′-nucleotidase and β-leucyl naphthylamidase were

Table 1
Effect of visible light and oxygen exposure of hepatocytes on permeability and enzymes of intracellular organelles

	Incubation (h)	Activity (nmol/min/mg protein)		% Activity	
		Initial	Total[a]	Total residual	Latent
Lysosomes					
N-acetyl β-glucosaminidase	2, dark	1 53	5.92	90	74
	2, light	4.14	5.33		5
Acid phosphatase	2, dark	0.76	1.61	88	53
	2, light	1.18	1.42		17
Cathepsin C	2, dark	2.32	5.35	18	56
	2, light	0.77	0.94		19
Peroxisomes					
Catalase	4, dark	7.68	15.36	2	50
	4, light	0.18	0.24		25
Mitochondria					
Cytochrome *c* oxidase	12, dark	37.55	99.30	107	62
	12, light	79.94	106 50		25

[a] Assays in the presence of Triton X-100 or Emasol detergents

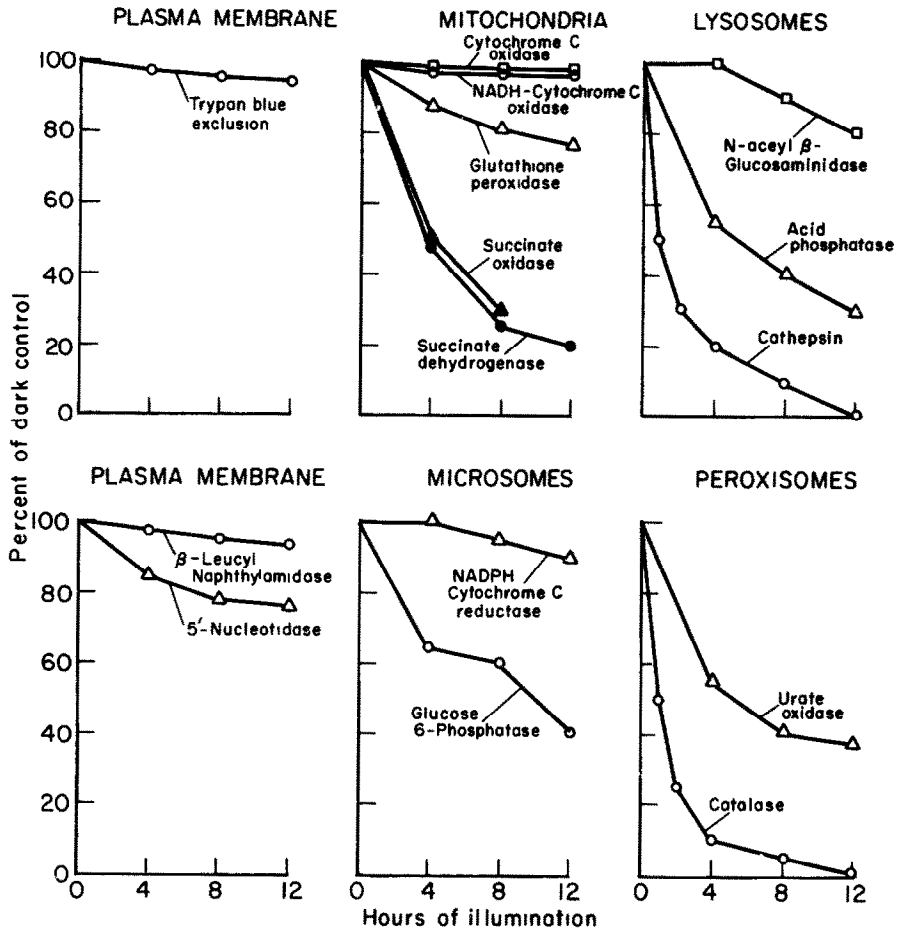

Fig.1 Effects of exposure of rat hepatocytes to visible light and oxygen

only slightly inactivated Under these conditions, however, other intracellular enzymes were markedly inactivated. Mitochondrial damage was indicated by a decrease in latency of cytochrome *c* oxidase and destruction of various enzymes in the following order succinate dehydrogenase > succinate oxidase > glutathione peroxidase > NADH-cytochrome *c* oxidase > cytochrome *c* oxidase (fig.1).

Lysosomal damage was also extensive, as indicated by release of cathepsin C, acid phosphatase and *N*-acetyl-β-glucosaminidase, indicating loss of latency of these enzymes. Following this, the total activity of these enzymes also diminished, indicating that they were being inactivated. Inactivation of these enzymes was in the same order as their loss of latency. Some evidence of damage to microsomal membranes was indicated by a decline in glucose-6-phosphatase activity. The most light-sensitive enzyme was found to be catalase, an enzyme associated with the peroxisomal fraction Another peroxisomal enzyme, urate oxidase, also present in the same subcellular compartment, was relatively less susceptible to light damage. In addition to inactivation of protein enzymes, destruction of membrane lipids was indicated by lipid peroxidation.

3.1. *Protection against visible light damage*

Several treatments were attempted to prevent visible light damage. The addition of succinate + KCN were maximally effective (fig.2). Also EDTA was

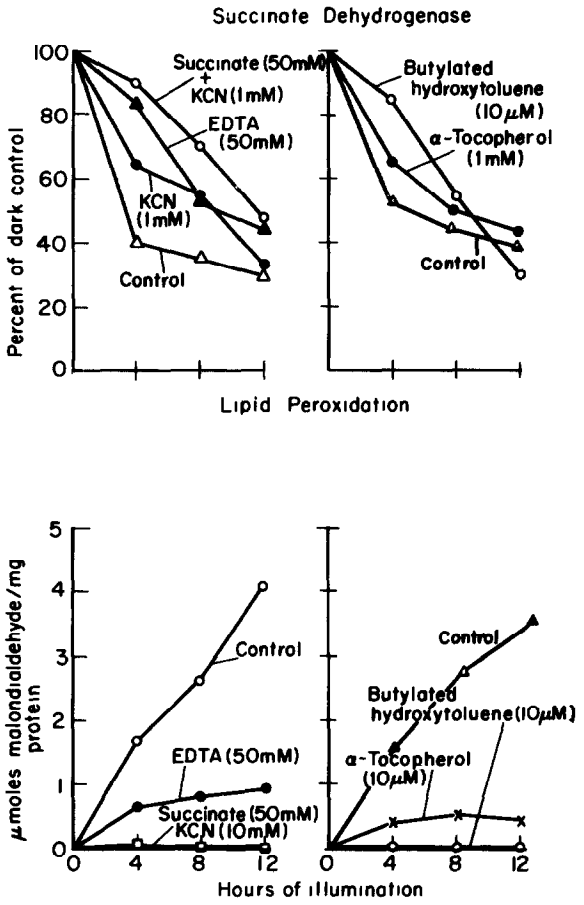

Fig.2 Protection of hepatocytes against visible light damage by addition of antioxidants and reducing reagents. In experiments involving antioxidants, controls were made with an equivalent amount of ethanol added.

active in suppression of enzyme inactivation as of succinate dehydrogenase and lipid peroxidation. Incubation of hepatocytes with succinate + KCN or EDTA are both conditions which would lead to extremely reducing conditions DL-α-tocopherol and butylated hydroxytoluene were very effective against prevention of lipid damage but only the latter antioxidant appreciably affected the pattern of enzyme inactivation. Rats fed with a vitamin E-deficient diet showed a marked increase in lipid peroxidation compared to rats on a vitamin E-supplemented diet (table 2). However, no significant difference in enzyme inactivation was observed.

Table 2
Effect of visible light on lipid peroxidation and succinate dehydrogenase in hepatocytes from rats fed on vitamin E-deficient and supplemented diets

Incubation time (h)	E-Deficient		E-Supplemented	
	(nmol malonaldehyde/mg protein)			
	dark control	light	dark control	light
2	0.37	3 20	0.40	2 08
4	0.50	5 64	0.80	2 73
8	0.79	6 10	0.42	3.75
12	0 69	6.22	0.42	3.75

	Succinate dehydrogenase activity	
	% act. dark control	% act dark control
2	75[a]	74[b]
4	69	64
8	41	43
12	23	29

[a, b] Zero time activity was 0 062 and 0 071 μmol succinate/mg protein, respectively

Data are averages of duplicate samples

4. Discussion

Visible light exposure clearly leads to a selective pattern of intracellular destruction. The plasma membrane is highly refractive to damage, whereas the most marked pattern of damage was the loss of the latency of lysosomal enzymes and their inactivation, and the inactivation of catalase. There have been previous reports indicating inactivation of catalase by sunlight [18,19] and we (Kellogg and L.P., unpublished results) will report separately upon the photoinactivation mechanism of catalase. The great lability of lysosomal enzymes in hepatocytes to photodamage is expected since these organelles are known to accumulate flavins, hemes and transition metals such as iron and copper [20]. Lysosomes also contain flavin hydrolysing enzymes such as acid phosphatase Hence flavin-photosensitized reactions may occur. Indeed, dye photosensitized damage to lysosomes has been observed [21] by fluorescence microscopy The pattern of inactivation of mitochondria in intact hepatocytes follows closely that observed earlier for

mitochondria in vitro where inactivation was largely confined to flavin-linked enzymes.

These results suggest that the location of flavin or heme photoreceptors in intracellular organelles and their relative absorption may determine the degree to which enzymes are inactivated. The protection against visible light damage of succinate dehydrogenase by substrate + KCN or by EDTA may be the result of flavin reduction which can occur photochemically under these conditions [22]. Vitamin E was highly effective in preventing lipid peroxidation but did not significantly protect against enzyme inactivation, which indicates that a different mechanism is involved in each case. The fact that cells from vitamin E-deficient rats showed augmented rates of lipid peroxidation without increased enzyme inactivation supported this idea.

The marked sensitivity of catalase and the loss of latency and inactivation of mitochondrial energy-linked enzymes suggest that crucial systems involved in the protection against oxidative damage in the cell will result in pathology and may result in photokilling. However the various enzymes involved in irreparable intracellular damage have not yet been unambiguously demonstrated, although damage in all three of the organelles involved in cellular oxidative and energy metabolism occur simultaneously. Future studies are being directed towards elucidating these enzymes and towards identifying the nature of the free radical species initiated by blue light exposure.

Acknowledgements

This research was supported by the Department of Energy and by a grant from Hoffman-La Roche, Inc , Nutley, NJ. We are also grateful to the University of Hong Kong and Professor T. Boyde for support of this research and to Drs B. Aggarwal and E. W. Kellogg, iii, for helpful discussions.

References

[1] Anwar, M. and Prebble, J. (1977) Photochem. Photobiol. 26, 475–481.
[2] Epel, B. L. (1973) Photophysiol. 8, 209–229.
[3] Stoien, J. D. and Wang, R J. (1974) Proc. Natl. Acad. Sci. USA 71, 3961–3965.
[4] Sulkowski, E., Geuria, B. and Defaye, J. (1964) Nature 202, 39.
[5] Litwin, J. (1972) J. Gerontol. 7, 381–386.
[6] Pereira, O. M., Smith, J. R. and Packer, L (1976) Photochem. Photobiol. 24, 237–242.
[7] Krebs, H. A., Cornell, N. M., Lund, P. and Hems, R. (1974) in: Regulation of Hetatic Metabolism (Lundquist, P. and Tygstrap, N. eds) pp. 726–750, Academic Press, New York.
[8] Aggarwal, B. B., Quintanilha, A. T., Cammack, R. and Packer, L. (1978) Biochim. Biophys. Acta 502, 367–382.
[9] Sun, A. S. S., Aggarwal, B. B. and Packer, L. (1975) Arch. Biochem. Biophys 170, 1–11.
[10] Morre, D. J. (1971) Methods Enzymol. 22, 137.
[11] Sanui, H. (1974) Anal. Biochem. 60, 489–504.
[12] Barber, A. A. (1966) Lipids 1, 146–151
[13] King, T. E. (1967) Methods Enzymol. 10, 322–331.
[14] Little, C., Olinscu, R., Reid, K. G. and O'Brien, P. J. (1970) J. Biol. Chem. 245, 3632–3636.
[15] Leighton, F., Poole, B., Beufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. and deDuve, C. (1968) J. Cell Biol. 37, 482–513.
[16] Kalckar, H. M. (1947) J. Biol. Chem. 167, 429–443.
[17] Peters, T. J., Miller, M. and deDuve, C. (1972) J Exp. Med. 136, 1117–1137.
[18] Mitchell, R. L. and Anderson, I. C. (1965) Science 150, 74
[19] Aronoff, S. (1965) Science 150, 72–73.
[20] Tappel, A. L. (1973) in. Lysosomes in Biology and Pathology (Dingle, J. T. and Fell, H. G. eds) vol 2, pp. 207–240, North-Holland, Amsterdam, London.
[21] Allison, A. C., Magnus, I A. and Young, M R. (1966) Nature 209, 874–878
[22] Massay, V., Stankovich, M. and Hemmerich, P. (1978) Biochemistry 17, 1–8