In Vivo Response of the Rat's Retinal Pigment Epithelium to Azide: Changes Induced by Light Damage

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Abstract Functional changes in retinal pigment epithelium (RPE) associated with light-induced retinal damage were studied by measuring transocular potential changes evoked by injections of azide and thiocyanate (SCN^-). The retinal damage by light in the rat is classified into two types: Type 1, rod cell death associated with RPE deterioration; Type 2, the loss of rod cells without RPE deterioration. To study the type 1 damage, littermate pairs of long-term dark-adapted adult albino rats were tested at 1 h and 10 d after the exposure to green light of 1,200 lx for 1/2 to 24 h. Time course of the damage progress was also followed for 12 h. We found that 1) RPE was affected rapidly by the damaging light, 2) the exposure length determined the ultimate degree of RPE damage, 3) damaging effects on RPE proceeded slower and weaker after exposure than during continuous light, 4) progress of the damage in RPE was two-phasic; during the first phase, the SCN^- response was enhanced and the azide response was reduced; both responses were decreased rapidly in the second phase. The first phase was assumed to indicate a depolarization of the basolateral membrane of RPE, and the second phase to manifest the structural deterioration of RPE. The type 2 damage was studied in young rats with exposure to weak light for 28 d. At 30 d after the exposure, a-wave of the ERG and number of rod cells were substantially reduced but azide and SCN^- responses were affected slightly.

Key words: azide, thiocyanate, retinal pigment epithelium, retinal light damage, rat.

The retinal damage by visible light in the rat has been described by many investigators. Interesting features of the damage are that the action spectrum of the damage is similar to the absorption spectrum of rhodopsin [1,2]; the damage is produced with light intensities that are lower by several orders of magnitude than

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required for the production of pathological reactions in any other tissues by visible or near UV light [1]; the threshold of retinal damage is given by light that bleaches *in vivo* rhodopsin by about 15%; retinal pigment epithelium (RPE) of either the albino or pigmented rat is similarly affected by the same retinal irradiation [1]. What then is the mechanism of the light damage of RPE?

The association of rod cell death with RPE destruction is not an essential feature of the damage; under certain conditions, RPE is left intact light-microscopically despite the substantial loss of rod cells. Noell, therefore, classified the damage into two types; namely, type 1 is a combination of rod cell death and RPE deterioration, and type 2 represents the loss of rod cells in association with gross RPE preservation [3].

The type 1 damage is histologically evident by a "lesion," an area of the retina where all rod cells and RPE have disappeared 1 to 2 weeks after an exposure to light. The lesion is surrounded by a narrow border zone with partially surviving rod cells and RPE. Beyond this border zone, the surviving retina shows almost normal histology. Size of the lesion varies from widths of less than 100 μm to extend over the whole retina depending mostly on the duration of light exposure at a given light intensity. Progress of the damage is suggested to be rapid because massive RPE swelling with chromatin dispersion and pyknotic nuclei of rod cells are evident at 4 h after the start of an exposure [1]. Conditions favorable for the development of type 1 damage are long-term dark adaptation for at least several days [4] and elevation of body temperature [1].

The type 2 damage tends to dominate in rats maintained in cyclic weak light. Day and night cycling is a main determinant of damage and cyclic light is suggested to prevent or reduce RPE pathology [3, 5]. The type 2 damage is especially well expressed in young rats exposed to relatively weak light for several weeks.

In order to elucidate the mechanism of RPE damage caused by light exposures, we applied the *in vivo* electrophysiological tests of RPE described in the preceding paper [6] combined with histological, biochemical, and electroretinographical assessment of retinal cell damage. Bolus injections of azide and thiocyanate (SCN^-) through a venous canula change the membrane potential of RPE cells and thus evoke the cornea-positivie azide response and cornea-negative SCN^- response, respectively. At present they are the only electrophysiological tests of the state of RPE function that can be applied during and shortly after the damaging exposure to light. We concentrated on determining the time course of development of RPE pathology in the type 1 damage and then compared the type 1 with the type 2 damage.

A preliminary report has been published elsewhere [7].

MATERIALS AND METHODS

The rats of this study were bred in the animal quarters of the Medical Center and were kept in almost continuous darkness starting postnatal day 7. All of them

were male and albino. For the study of the type 1 damage, CD rats originated from Charles River Laboratories of ages between 4 to 6 months were used. For the study of the type 2 damage, inbred normal rdy^+ rats of LaVail were used in comparison with heterozygotes obtained by crossing of the rdy^+ rats with LaVail's congenic rdy^- rats expressing hereditary retinal dystrophy.

The rats were exposed to light in cylindrical wire-mesh chambers surrounded by 1/8 inch thick green plastic plate (Plexiglass, #2092) transmitting lights of 490–580 nm range. Three circular 32 W fluorescent lamps (GE, Cool White) were stacked around the green plastic. For the study of type 2 damage, white translucent paper was inserted between the plastic and the lamps to reduce light intensity. At the center of each chamber was a smaller wire cage of about 10 cm in diameter housing mostly only one rat. Illumination in this area was about 1,200 lx without the white translucent paper and 260 lx with the paper. The chambers were closely matched with regard to illumination, and temperature was kept close to 23°C by ventilating each chamber separately. Water and diet were continuously supplied. The rats were placed in the chambers without prior exposure to environmental light, and all exposures commenced at around 9 AM. After the exposures, the rats were either immediately prepared for electrophysiological recordings or kept in complete darkness for 10 or 30 d. Before the recordings, the rats were anesthetized with urethane (1 g/kg) under dim red light.

Electroretinogram (ERG) was evoked by a xenon flash (Grass, PS2) and was recorded by using conventional electrodes and an a.c. amplifier [1]. The azide- and SCN^- -induced transocular (d.c.) potential changes were measured as described in the preceding paper [6]. Volume of injected boluses was 0.4 ml. The concentrations of sodium salts of azide and SCN^- were 15 and 130 mM, respectively.

To assess the retinal cell loss, DNA content in whole retina was measured. Method to measure DNA concentration [8] was adapted for the rat retina. After isolating the whole retinas, individual retinas were homogenized, fluorescent DNA-indicator (Hoechst, #33258) was added, and then DNA content were determined by Farrand fluorometer (360 nm excitation and 420 nm emission).

From the DNA content in a whole retina, the average DNA content in retinas of hereditary dystrophy at advanced stages (27% of normals) was subtracted. The result reflects the content of DNA derived mostly from rod cells, because the dystrophic retina lost virtually all rod cells while cells in the inner layers have survived morphologically as well as functionally [9, 10]. The rod cell DNA content was expressed in % of the normal controls not exposed to light.

All experiments were terminated by dissection of one eye for histology, or both if DNA measurement was not performed. The eye was fixed in Zenker's solution, paraffin embedded, sectioned at 4 vertical planes and hematoxylin-stained.

RESULTS

Type 1 light damage, 10 d after exposure

The rats were exposed to about 1,200 lx green light for 1/2 to 24 h, then kept in the dark for 10 d before the electrophysiological and histological examinations. We observed the characteristic retinal lesion where photoreceptor cells and RPE had almost completely disappeared, confirming that the damage was of type 1. Size of the lesion was dependent on duration of the exposures. The histological manifestation of the type 1 damage of the rat's retina has been extensively described [1, 3, 11].

Figure 1 shows relations between duration of the exposure and peak amplitudes of the azide and SCN^- responses. Following an exposure for only 2 h, amplitudes of the azide and SCN^- responses were reduced to 10% of those of control rats of the same litters. No responses were detectable when exposure duration was as long as 12 and 24 h.

Fig. 1. Relation between the response originating from RPE and the duration of light exposure in type 1 damaged retina of adult long-term dark-adapted rats. Rats were exposed to green light of about 1,200 lx for 0.5 to 24 h. The responses to the bolus injections of 15 mM NaN_3 (A) and 130 mM NaSCN (B) were measured 10 d after the light exposure. Peak amplitudes of the responses relative to the unexposed controls are plotted against the exposure duration. Data are expressed as the mean $\pm$ standard deviations from at least four rats except for the exposure of 0.5 h.

Fig. 2. Correlations of the ERGs and RPE responses in type 1 damaged adult rat retinas of various severity. ERG was recorded prior to the measurements of RPE responses. Data are obtained from the same animals used to obtain data shown in Fig. 1. A: Correlation of the ERG a-wave elicited by the maximal flash to the b-wave in response to the flash attenuated by a neutral density filter of 4 log units. B: Correlation of the azide response with the ERG a-wave. C: Correlation of the azide response with the ERG b-wave. D: Correlation of the azide response with the SCN⁻ response.

Also affected were the ERG waves of the exposed rats recorded just before the azide and SCN⁻ response measurements. Figure 2 shows the relations among the amplitudes of a- and b-waves of ERG, azide and SCN⁻ responses. The b-wave was recorded at first by a flash of light attenuated by 4 log units. Then, the a-wave was elicited by a flash of the maximal intensity. ERG measurements were made on all rats used in the study shown in Fig. 1. Because exposure duration varied over a wide range, the amplitudes of responses ranged from zero to normal. The b-wave amplitude was linearly related to the a-wave, the correlation coefficient, $r = 0.919$ (Fig. 2A). This correlation is consistent with a previous study [3]. Figures 2B and C provide the new findings that the peak amplitude of the azide response was linearly related to that of a- and b-waves. A straight line also fits the data for the azide response in relation to the SCN⁻ response (Fig. 2D). Results indicate that an

Fig. 3. Relationship between peak azide response and the retinal content of rod cell DNA in type 1 damaged retinas of various severity. Ten days after the exposure, the azide response was recorded, then the same eye was immediately processed to measure the retinal content of rod cell DNA. The three points to the right are from unexposed littermates.

exposure to light for a given duration affected these 4 responses almost equally though the generation mechanisms for these responses are different. A common factor, probably size of the retinal lesion, determines the magnitude of decrease in these responses. In the next section, we studied the relation between the size of the lesion and amplitude of the azide response.

Type 1 damage, relationship between azide response and rod cell DNA content

Histological measurements of the damaged retina verified that the rod cell DNA content in a whole retina is a measure of the remaining retinal area where rod cells and RPE were left intact. Amount of rod cell DNA decreased, therefore, reflects the size of the retinal lesion. At 10 d after the exposure, the azide response was recorded and the DNA content was determined from the same eye. As shown in Fig. 3, amplitude of the azide response was non-linearly related to the rod cell DNA; a mild DNA loss of around 25% was associated with 50% reduction in the azide response.

Type 1 damage, measured 1 h after exposure

Acute effects of the damaging exposure to light were also studied. Littermate pairs of the rats, used in the experiment shown in Fig. 1, were tested 1 h after the same exposures. Procedure was exactly the same as for the companion rats tested after 10 d. As shown in Fig. 4A, almost complete loss of the azide response was observed when the exposure duration was longer than 12 h. Damage was as severe

Fig. 4. RPE responses measured 1 h after light exposure. One hour after the light exposure, the azide and SCN⁻ responses were recorded from littermates of the rats used in Fig. 1. Data are expressed as the mean $\pm$ SD from at least four rats except that of 1-h exposure.

as demonstrated by tests done 10 d after the exposures. For shorter durations, however, a substantial fraction of the azide response was still preserved. It indicates that the light-induced pathological processes proceeded further even in the dark.

Except for exposures of 12 and 24 h, the SCN⁻ responses were better preserved than the azide response (Fig. 4B). In contrast, at 10 d after the exposures, both responses were almost equally affected. The average SCN⁻ response after 4 h exposure was even larger than the controls. It seems as if there are two phases in the development of the RPE damage: the initial one with reduced azide but enhanced SCN⁻ responses, and the second one manifested by the loss of both responses.

Type 1 damage, repeated testing after exposure

In order to obtain a detailed account of the development of the RPE pathology, rats were tested repeatedly at intervals of 1–2 h following exposures for 2, 6, and 12 h. Results displayed in Fig. 5 reveal 1) that the RPE damage proceeded in the dark; and 2) that following 2-h exposure, SCN⁻ responses were enhanced above control for about 5 h, while the azide responses were reduced. The latter characterizes the first phase of the RPE damage described above. The second phase characterized by the rapid loss of both responses dominated after 7 to 10 h

Fig. 5. Time course of RPE responses loss after exposure. Azide and SCN^- responses of individual rats were measured repeatedly. Graphs 1: changes in the peak amplitudes of azide and SCN^- responses after 2-h exposure. Graphs 2: changes in the peak amplitudes after 6-h exposure. Data points 3: the peak amplitudes of azide and SCN^- responses measured 1 h after a 12-h exposure. The peak amplitudes of the responses are expressed in % of the unexposed controls.

following the beginning of exposures. The rapid loss of both responses suggests that the structural deterioration of RPE was occurring. All points on graphs labeled as 2 (6-h exposure) are lower than those of graph 1, indicating that the deterioration process proceeded faster in continuing light than in the dark. The process seemed to have proceeded to completion during the 12-h exposure (data points 3). During the phase of rapid loss in amplitude, the SCN^- responses were consistently higher than azide responses indicating that the first phase of damage was apparently overlapping with the second phase. This may indicate that RPE deterioration did not proceed simultaneously over the whole retina or susceptibility to light was not uniform over the retina.

Table 1. Effects of constant weak light for 3 weeks on young rats in % of control values $\pm$ SD.

Azide response	SCN ⁻ response	a-wave	b-wave	DNA rod cells
84.3 $\pm$ 15.1	70.0 $\pm$ 15.4	21.4 $\pm$ 5.4	43.8 $\pm$ 10.6	40.3 $\pm$ 7.3

$n = 10$. a-wave was elicited with maximum flash intensity; b-wave was elicited with 1/10,000 maximum flash intensity. SD of control value is $\pm 15.3\%$ for the azide and $\pm 11.3\%$ for the SCN⁻ response.

Type 2 light damage

The type 2 light damage is characterized by rod cell death with simultaneous sparing of RPE [3]. We studied the congenic normal *rdy*⁺ strain of LaVail in comparison with heterozygotes obtained by crossing of the normal *rdy*⁺ rats with the abnormal *rdy*⁺ rats. Since we observed no differences in the susceptibility to light between them [12], results from both kinds of rats were collected together.

The rats were exposed to about 260 lx green light for 28 d starting at the age of 21 d. Following the exposure, the rats were kept in a constant dark environment for 30 d. Their a- and b-waves of ERG, azide and SCN⁻ responses were then tested. After testing, one eye of every rat was used to measure the rod cell DNA, the other eye was used for histological study. Histology showed widespread rod cell death, especially in the dorsal half of the retina. RPE was preserved throughout the retina except for one small spot of a lesion in one rat.

As listed in Table 1, the amplitudes of azide and SCN⁻ responses were slightly reduced. In contrast, the averaged a-wave amplitude was very low. As is typical for the type 2 damage [3], the b-wave was relatively well preserved. On the average, 60% of the rod cell DNA of a whole retina had disappeared. Results indicate that a substantial loss of rod cells, as determined by ERG, DNA measurement and histology, was associated with the better preservation of RPE as evidenced by the electrical responses of RPE origin.

DISCUSSION

A key to understand the retinal damage in the rat is that the action spectrum of the damage coincides with the absorption spectrum of rhodopsin. This was first documented by Noell *et al.* [1], then by Williams and Howell who used more elaborate methodology [2]. In the study by Noell *et al.* [1], the damage was of type 1 manifested by a confluent area where the rod cells and the RPE had disappeared; in the paper by Williams and Howell, the damage was expressed by a distributed loss of outer nuclei, indicating that the damage was probably of type 2. Therefore, both types of damages are rhodopsin-mediated and the loss of RPE should be secondary to the action of light on the rod cells (see [13]).

By using the light-independent azide and SCN⁻ responses, our study demon-

strated that the effect of damaging light on RPE occurs rapidly. A definite temporal correlation between the damaging effects on RPE and rod cells cannot be made, but there are suggestions that the time courses of both effects are similar. For instance, Noell *et al.* [1] repeatedly recorded the ERG after 1-h exposure to a rat whose body temperature was elevated to accelerate the damaging processes. The ERG recovered to about 40% of the control within the first 3 h manifesting rhodopsin regeneration. Thereafter, the ERG declined steadily to reach a level of about 25% at 6 h after and 10% at 7 d after the exposure. This is about the same ERG effect as observed in the present study with 2-h exposure and the time course of deterioration is comparable to that of the azide response.

The present study revealed two phases in the progress of the damaging effects on RPE. The first phase was characterized by a moderate decrease in the azide response coupled to an increase in the SCN^- response. Based upon the interpretation of the responses described in the preceding paper [6], it is assumed that the first phase represents depolarization of basal membrane of RPE while the structural integrity of RPE is still preserved.

The depolarization of basal membrane of RPE has been described in a variety of conditions. It occurs during hypoxia and hypercapnia [14]. It can be caused by the intravenous injection of hypertonic solution [15]. Furthermore, the slow positive wave of the d.c. potential, the light peak of ERG, has also shown to be a basal membrane depolarization [14, 16]. For the generation of this light peak, some chemical agents, which depolarize the basal membrane of RPE, are suggested to be delivered from photoreceptors into the subretinal space [17–20]. It is tempting to speculate that the light peak may be a (sub-threshold) manifestation of a potentially damaging reaction to light in the rat.

Repeated brief exposures for short periods of time have been shown to enhance the damage compared with continuous light [1]. The question arises whether the shifts from light to darkness have a specific potentiating effect. Repeated measurements of the azide and SCN^- responses after exposures gave no evidence for this possibility.

The ERG's a- and b-waves, the azide and SCN^- responses were proportionally decreased when measured 10 d after the exposures causing type 1 damage. Size of the retinal lesion, where rod cells and RPE have disappeared, seemed to define the magnitude of decreases in these responses. Currents generated by surviving area of the retina would be shunted by the lesion where the high resistance barrier of RPE had been lost.

Quite different results were obtained from the study for the type 2 light damage. As a result of preservation of RPE, the azide and SCN^- response were affected only slightly. On the other hand, the a-wave was reduced to about 20% of the controls and more than 50% of the rod cell DNA had disappeared. The greater decrease in the a-wave than the b-wave in type 2 damage probably indicates that the damage is confined to the outer retinal layers [3, 21].

At present, it cannot be answered whether the pathological RPE reaction in

the rat is secondary to events initiated in the rod cells. Cyclic light exposure of about 6 lx is reported to affect the function of RPE in albino rats without impairment of the photoreceptor functions [22]. However, a prolonged light exposure, sufficient to abolish RPE in normal rats, does not affect the retinas of hereditary retinal dystrophy at advanced stages [10]. These retinas contain virtually no rod cells while RPE and cells in the inner layers are light-microscopically left intact. It indicates that the presence of rod cells is necessary to induce the damage in RPE. Therefore, the secondary involvement of RPE is more probable than the alternative view that light exerts the "toxic" effects directly on RPE.

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