Biochimica et Biophysica Acta, 640 (1981) 231—239

BBA 79064

PHOTODYNAMIC ALTERATION OF CORNEA ENDOTHELIUM

RELATION TO BICARBONATE FLUXES AND OXYGEN CONCENTRATION

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(Received May 29th, 1980)

Key words: Photodynamics; Bicarbonate flux; Photooxidation; Catalase; Oxygen concentration; (Cornea endothelium)

Summary

Corneas were mounted in flux chambers and endothelial bicarbonate fluxes were determined following sensitization of endothelial cells with $5 \cdot 10^{-6}$ M rose bengal and exposure to light. Corneas exposed to light demonstrated an increased passive bicarbonate flux compared to corneas not photosensitized. Active bicarbonate flux was reduced after 5 min of light exposure, but not after 1 min of light exposure. The increase in passive bicarbonate flux was prevented by the addition of 200 μg/ml catalase to the bathing solution; however, catalase had no effect on the photodynamic alteration of active flux. Neither 10 mM ascorbic acid nor 1.012 g/l glutathione prevented the photodynamically induced increase in passive flux. Perfusion of corneas with $5 \cdot 10^{-6}$ M rose bengal dissolved in a sucrose-substituted Krebs-Ringer bicarbonate solution with a p_{O_2} of 124 ± 4.0 mmHg and exposed to light swelled at rates more rapid than corneas treated in a similar fashion but perfused with a solution with a p_{O_2} of 20 ± 4.6 mmHg. This study demonstrates that photodynamically induced corneal endothelial cell alteration results in increased passive bicarbonate flux, a time-dependent decrease in active bicarbonate flux, is oxygen dependent, and is at least in part secondary to H_2O_2 produced by the dismutation reaction of the superoxide free radical.

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Introduction

Cornea endothelial cells perfused in vitro with rose bengal and exposed to light undergo an alteration of physiologic function resulting in corneal swelling [1]. The corneal swelling rate is dependent on both rose bengal concentration and duration of light exposure. It is probable that this photodynamic effect on cornea endothelial cells is secondary to H_2O_2 produced during the dismutation reaction of the superoxide free radical since catalase, but not superoxide dismutase, prevents the corneal swelling associated with this phenomenon [1].

The method by which photon absorption by presensitized membranes results in their physiologic alteration is not entirely understood. The excited photosensitizer molecule usually causes a photooxidation reaction mediated through its triplet state. The sensitizer may interact with another molecule directly (type I process) or may involve transfer of the excitation from sensitizer to O_2 (type II process) producing either singlet oxygen or the superoxide anion [2—5]. Recent work has indicated that photodynamically induced membrane modification by the fluoresceins (of which rose bengal is a member) is at least in part mediated by singlet oxygen [6]. Singlet oxygen may play a role in photooxidation reactions, but it is strongly suspected that in the corneal endothelial cell model, H_2O_2 produced during the dismutation reaction of the superoxide free radical plays an important role [1].

It has been established that a net bicarbonate flux exists across the corneal endothelium from the stromal facing side to the aqueous facing site [7—10], and that a bicarbonate-dependent ATPase has been found in the corneal endothelium [11]. Since it is probable that the endothelial bicarbonate flux is related to water movement out of the corneal stroma for the maintenance of corneal clarity, and since O_2 may be involved in the mediation of the photodynamic alteration, it was the purpose of this investigation to determine how corneal endothelial bicarbonate fluxes are altered during in vitro perfusion with rose bengal and exposure to light and to relate the photodynamic alteration to O_2 concentration.

Methods

Group I. Albino rabbits of either sex weighing 2.5—3.5 kg were killed with an overdose of sodium pentobarbital, each corneal epithelium was removed and the cornea was mounted in a flux chamber as previously described [8—10]. In a darkened room, $5 \cdot 10^{-6}$ M rose bengal was dissolved in bicarbonate Ringer solution containing 0.134 g/l adenosine, and 0.092 g/l glutathione [12,13], and was introduced into paired chambers facing the endothelial surface: Ringer solution without rose bengal was on the epithelial surface. Illumination from a 25 W incandescent light at a distance of 5 cm was passed through the epithelial surface chamber for either 1 or 5 min. Previous work has documented a lack of temperature rise of the preparation when exposed to light under similar conditions [1]. Following light exposure, the chamber facing the endothelial surface was flushed with 5 ml of bicarbonate Ringer solution containing adenosine and glutathione. $H^{14}CO_3^-$ in bicarbonate Ringer solution with adenosine and gluta-

thione was then introduced into the chambers as previously described [8—10] for a 1 h equilibration, and HCO_3^- fluxes measured at 30-min intervals over the succeeding 3 h. Unidirectional endothelial bicarbonate fluxes were calculated as shown previously [8,10], and the flux rate expressed as μequiv./cm^2 per h. The corneal surface area used in the calculations to express flux rate was the 'posterior corneal spherical area' [10], and not the chamber plane across sectional area as in two prior publications [8,9]. Control corneas were bathed with rose bengal on the endothelial surface as were experimental corneas, but were not exposed to light.

Group II. Corneas were mounted as described above but were bathed on the endothelial surface with 200 μg/ml catalase, and $5 \cdot 10^{-6}$ M rose bengal, dissolved in bicarbonate Ringer solution with added adenosine and glutathione during light exposure. Subsequent to light exposure, the chamber was flushed and the endothelium bathed for 3 h with $H^{14}CO_3^-$ in bicarbonate Ringer solution with added adenosine and glutathione as in Group I.

Group III. Same as Group II except 10 mM ascorbic acid was used instead of catalase.

Group IV. Same as Group II (except no catalase), however, the glutathione concentration in the Ringer solution was increased to 1.012 g/l.

Group V. Eyes were removed from the animals, and corneas were prepared and mounted in the specular microscope [12,14—16]. The endothelial surface was perfused with a modified Krebs-Ringer bicarbonate solution in which 5 g/l sucrose replaced glucose. The solution also contained reduced 0.092 g/l glutathione and 0.134 g/l adenosine [12,13,16,17]. Perfusion was performed at 37°C and 15 mmHg pressure and silicone oil was placed on the epithelial surface.

After a 1 h stabilization period and the recording of corneal thickness, the room lights were shut off. Experimental corneas were perfused with $5 \cdot 10^{-6}$ M rose bengal dissolved in sucrose-modified Krebs-Ringer bicarbonate solution with added adenosine and glutathione which had previously been bubbled for 45 min with O_2-free N_2. Subsequently, 0.001% pyrogallol was added, the solution bubbled an additional 10 min with O_2-free N_2, the pH adjusted to approx. 7.3 and the solution placed in a sealed syringe. Control corneas were perfused with $5 \cdot 10^{-6}$ M rose bengal dissolved in sucrose-modified Krebs-Ringer bicarbonate solution with added adenosine and glutathione and with an ambient O_2 concentration [1]. In order to facilitate complete replacement of the perfusing solution with the rose bengal solution in the perfusion chambers, the perfusion rate was increased to allow for a flow of 10 ml in the first 6 min and was then reduced to 3.2 ml/h. Experimental and control corneas were exposed to a 25 W incandescent light at a distance of 5 cm for 1 min. A water-filled petri dish was placed between the light bulb and the cornea to absorb heat generated by the lamp [1]. Following exposure to light, the experimental corneas were perfused for 2 h in the dark with sucrose-modified Krebs-Ringer bicarbonate solution with added adenosine and glutathione which had previously been bubbled for 45 min with O_2-free N_2, 0.001% pyrogallol added, bubbled an additional 10 min with O_2-free N_2, the pH adjusted to approx. 7.3 and placed in a sealed syringe. Control corneas were perfused for 2 h in the dark with sucrose-modified Krebs-Ringer bicarbonate solution with added adenosine and gluta-

thione and with an ambient O_2 concentration. The perfusion rate of experimental and control corneas was 2 ml in the first 1 min following rose bengal perfusion to allow for washout of the rose bengal solution and was then reduced to 3.2 ml/h for the remainder of the experiment. Corneal thickness was recorded every $\frac{1}{2}$ h for a total of 2 h after perfusion with rose bengal and exposure to light. The change in corneal thickness was determined by linear-regression analysis on six corneas per experimental group and a comparison of experimental and control regression lines was made by analysis of covariance. Variance in the data was expressed as the mean ±95% confidence limits [18, 19].

Following the 2 h perfusion, syringes containing the perfusion solution were sealed and p_{CO_2}, p_{O_2} and pH were determined on a blood microanalyzer (BMS 3 MK2 microsystem, pH M72 Acid Base analyzer). Bicarbonate content was calculated using the Henderson-Hasselbach equation.

It was also determined if O_2-deficient sucrose-modified Krebs-Ringer bicarbonate solution with added adenosine and glutathione had an untoward effect on corneal endothelium. Experimental corneas were perfused for 3 h in the specular microscope with sucrose-modified Krebs-Ringer bicarbonate solution with added adenosine and glutathione which had been bubbled for 45 min with O_2-free N_2, 0.001% pyrogallol added, bubbled an additional 10 min with O_2-free N_2, the pH adjusted to approx. 7.3 and placed in a sealed syringe. Paired control corneas were perfused for 3 h with sucrose-modified Krebs-Ringer bicarbonate solution with added adenosine and glutathione with an ambient O_2 concentration. The p_{CO_2}, p_{O_2} and pH were determined at the end of the experiment and the bicarbonate content was computed.

Results

Group I (rose bengal and light)

Cornea endothelial bicarbonate passive fluxes (J_{epi}^{endo}) were greater in the corneas exposed to light than those kept in the dark for similar time periods in the presence of $5 \cdot 10^{-6}$ M rose bengal ($P < 0.01$). Passive fluxes (J_{epi}^{endo}) of corneas exposed to light for 5 min were significantly greater than those exposed to light for 1 min. Active bicarbonate fluxes (J_{endo}^{epi}) were reduced after 5 min of light exposure but were not reduced after 1 min of light exposure. With 5 min of light exposure the reduction of active flux (J_{endo}^{epi}) combined with the increase of passive flux (J_{epi}^{endo}) resulted in a marked reduction of net flux under conditions of photosensitization (Table I, group I). 1 min of light exposure resulted in a reduction of net flux because of the increase in passive flux.

Group II (rose bengal, light, and 200 μg/ml catalase)

Cornea endothelial bicarbonate passive fluxes (J_{epi}^{endo}) were significantly lower in the presence of catalase when corneas were bathed with rose bengal and exposed to light than in those corneas otherwise treated the same but not bathed with catalase. The passive flux in the presence of catalase under conditions of rose bengal perfusion and light exposure (1.99 ± 0.06 μequiv./cm^2 per h) was similar to that found in corneas bathed with rose bengal but not exposed to light (1.93 ± 0.10 μequiv./cm^2 per h). Catalase was ineffective in

restoring active fluxes to non-photosensitized levels (c.f. groups I and II, Table I).

Group III (rose bengal, light, 10 mM ascorbic acid)

Cornea endothelial bicarbonate passive fluxes (J^{endo}_{epi}) in the presence of ascorbic acid, rose bengal, and light were statistically similar to those bathed without ascorbic acid under otherwise similar conditions (c.f. groups I and III, Table I). Active fluxes (J^{epi}_{endo}) were similar to those found in non-photosensitized corneas.

Group IV (rose bengal, light, 1.012 g/l glutathione)

Cornea endothelial passive bicarbonate fluxes (J^{endo}_{epi}) in the presence of 1.012 g/l glutathione, rose bengal and light were significantly higher than those found under conditions of bathing with rose bengal and exposure to light in the absence of 1.012 g/l glutathione: 2.69 ± 0.09 as against 2.30 ± 0.09 ($P < 0.01$).

Group V (oxygen dependence)

Corneas perfused with rose bengal dissolved in Ringer solution with ambient O_2 content (Table II, subgroup A) swelled more rapidly after light exposure than those perfused with rose bengal dissolved in Ringer solution with a low p_{O_2} value (Table II, subgroup B). Corneas perfused with low p_{O_2} Ringer solution without rose bengal (Table II, subgroup D) swelled more rapidly than corneas perfused with Ringer solution with ambient O_2 content (Table II, subgroup C). The addition of rose bengal to low p_{O_2} Ringer solution (subgroup B) and exposure to light resulted in a corneal swelling rate of 13 ± 5 μm/h which was not statistically different from the 9 ± 2 μm/h swelling rate of subgroup D corneas perfused with low p_{O_2} Ringer solution alone and not photosensitized (Table II).

TABLE I

Cornea endothelial unidirectional bicarbonate fluxes (μequiv./cm² per h) during $5 \cdot 10^{-6}$ M rose bengal perfusion and exposure to 25 W incandescent light at 5 cm. All values are means ±S.E.

Duration of light exposure (min)	Group	Experimental conditions	n	J^{epi}_{endo}	J^{endo}_{epi}	J_{net}
5	I	light	36	3.66 ± 0.14 *	2.30 ± 0.09 ***	1.36
		no light	30	4.10 ± 0.17 *,**	1.93 ± 0.10	2.17
5	II	light + 200 μg/ml catalase	30	3.74 ± 0.01 **	1.99 ± 0.06	1.75
5	III	light + 10 mM ascorbic acid	24	4.04 ± 0.15	2.37 ± 0.09 ***	1.67
5	IV	light + 1.012 g/l glutathione	24	3.90 ± 0.18	2.69 ± 0.09 ***	1.21
1	V	light	30	4.07 ± 0.15	1.99 ± 0.06 ***	2.08
		no light	36	3.97 ± 0.12	1.72 ± 0.05	2.25

* $P < 0.05$.
** $P < 0.05$.
*** Different from corneas in group not exposed to light; $P < 0.01$.

TABLE II

RELATIONSHIP OF CORNEAL SWELLING RATE TO p_{O_2} AND PHOTOSENSITIZATION REACTION

Corneal swelling rates are given ±95% confidence limits, all other values are the means ±S.E.

Subgroup	n	Corneal swelling rate (μm/h)	p_{CO_2} (mmHg)	p_{O_2} (mmHg)	pH	HCO_3^- (mM)
(A) Rose bengal in normally oxygenated Ringer solution. 1 min light exposure	6	26 ± 6 *	19.1 ± 4.1	123.6 ± 4.0	7.42 ± 0.06	11.6 ± 1.5
(B) Rose bengal in low O_2 content Ringer solution. 1 min light exposure	6	13 ± 5 *,**	50.2 ± 2.4	20.3 ± 4.6	7.10 ± 0.25	15.6 ± 0.7
(C) Normally oxygenated Ringer solution	6	4 ± 2 ***	24.3 ± 3.3	113.7 ± 2.6	7.42 ± 0.04	15.2 ± 0.8
(D) Low O_2 content Ringer solution	6	9 ± 2 **,***	55.5 ± 5.3	28.0 ± 2.8	7.08 ± 0.04	15.9 ± 0.3

* Swelling rate subgroup A greater than group B; $P < 0.05$.
** Swelling rate subgroup B similar to group D; $P > 0.05$.
*** Swelling rate subgroup D greater than group C; $P < 0.05$.

Discussion

It has been found that corneas bathed on the endothelial surface with rose bengal in the specular microscope and exposed to light swell whereas those not exposed to light do not swell [1]. In addition, the photosensitized corneas do not undergo anatomic changes as determined by scanning electron microscopy [1]. The swelling rate was directly proportional to both rose bengal concentration and duration of light exposure. In this experiment it was found that the passive component (J_{epi}^{endo}) of the net cornea endothelial bicarbonate flux was increased in corneas bathed with rose bengal and exposed to light when compared to those not exposed. In addition, the passive flux was higher following 5 min of light exposure than that following 1 min of light exposure. The active flux was reduced after 5 min of light exposure, but not after 1 min of light exposure, demonstrating that the active component is apparently less sensitive than the passive component. The net flux was reduced more after 5 min of light exposure than after 1 min of light exposure because of a more pronounced effect on passive and active flux after 5 min. It is known that photosensitization reactions can cause alterations of membrane permeability and active transport as well as accelerate the rate of Na^+ uptake and K^+ loss [20,21].

In a previous experiment it was determined that 200 μg/ml catalase, but not 100 μg/ml superoxide dismutase, prevented the corneal swelling after exposure to rose bengal and light [1]. In the present experiment, 200 μg/ml catalase prevented the alteration of the passive endothelial bicarbonate flux, but had no effect on the alteration of active flux (Table I). This gives added support to the concept that the photodynamic functional alteration of cornea endothelium is at least in part from the H_2O_2 produced during the dismutation reaction of the superoxide free radical. It is unclear why catalase did not block the alteration of active flux, but may reflect an inability of catalase to reach a specific transport site or a singlet oxygen effect. It is known that singlet oxygen can cause sodium channel block [6]. It is of interest that the free radical scavengers, 10 mM ascorbic acid (which is similar to the aqueous humor concentration) and 1.012 g/l glutathione, did not prevent photodynamic alteration of cornea endothelial passive bicarbonate fluxes. It is not understood why 1.012 g/l glutathione caused an increase in passive permeability to bicarbonate, since previous work under different conditions has shown that 0.092 g/l glutathione reduces passive bicarbonate permeability [10].

The results of this experiment also demonstrate that the photodynamic alteration of corneal endothelial cells is dependent upon O_2 concentration, and that a low O_2 content reduces the rate of corneal swelling following incandescent light exposure of rose bengal-presensitized corneas. The low O_2 content of group D control corneas resulted in a slight increase in swelling rate compared to control group C corneas that were perfused with normally oxygenated Ringer solution. This would be expected because a reduction of O_2 concentration will adversely affect any transport system. The photosensitization in group A in the presence of normally oxygenated Ringer solution markedly increased the rate of swelling, but the photodynamic effect was reduced in group B in the presence of low O_2 content Ringer solution to levels similar to

that of group D that were not photosensitized ($P > 0.05$). This series of experiments was performed using a sucrose-modified Krebs-Ringer bicarbonate solution which slightly stressed the endothelial cells. Experiments performed with Krebs-Ringer bicarbonate solution containing glucose did not demonstrate statistically significant differences in swelling rates of presensitized corneas with varying O_2 content. It is possible that yet a lower p_{O_2} may have produced a difference when perfusing with a glucose-containing solution, but this was not possible when working within the constraints of this model. The difference in swelling rates cannot be ascribed to variations in p_{CO_2} since the bicarbonate concentration is the most important determining factor [22]. The data in Table II also indicate that subgroups A and C have similar p_{CO_2} concentrations yet vastly different swelling rates, thus low p_{CO_2} did not affect corneal swelling rate. Previous work has demonstrated a reduced photodynamic effect in frog muscle and squid giant axons under conditions of reduced O_2 content [23,24]. In addition to O_2 dependence, the endothelial cell physiologic modification induced by photodynamic alteration is directly dependent on both dye concentration as well as duration of light exposure [1].

The precise mechanism has not been determined as to how photodynamic change affects endothelial cell function. It is known that the superoxide anion and photodynamic alteration are capable of perturbing lipid bilayers sufficiently to cause leakage of anions as has been shown in this experiment as well as in previous work [25]. Photosensitization reactions can also lead to sodium channel block possibly by lipid peroxidation, photooxidation of certain amino acid residues, and cross-linking of membrane proteins [26]. The mechanisms of photochemical change of cornea endothelial cells is the subject of continuing investigation and the corneal endothelial model may prove useful for the study of anatomical and functional relationships.

Acknowledgements

This work was supported in part by Research Grants EY02386 (to D.S.H.) and EY01413 (to K.G.) from the National Eye Institute, in part by a Research Grant from the Georgia Lions Lighthouse Foundation, Inc., and in part by the Lions Clubs of Augusta, GA. A Wang 2200 Computer, used for statistical data evaluation, was provided through a Research to Prevent Blindness, Inc., grant.

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