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OXY RADICALS IN THE EYE TISSUES OF RABBITS AFTER DIQUAT *IN VIVO*

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It is our hypothesis that oxygen free radicals are the triggering agents in cataractogenesis. However, besides H_2O_2 there is no direct evidence of generation of oxy radicals in the eye tissues. Due to extremely short life of $O_2^{\cdot-}$ and $OH\cdot$ it is not possible to measure their cellular steady state levels. We found that indirect spectrophotometric techniques based on superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction for estimation of $O_2^{\cdot-}$, salicylate hydroxylation for $OH\cdot$ and peroxidase catalysed reoxidation of 2,6-dichlorophenolindophenol for H_2O_2, were suitable, sensitive and reproducible for measurements of the reactive species of O_2 produced in the eye tissues by oxy radical enhancer, diquat in the rabbit eye *in vivo*. After a single intravitreal injection of 60, 120 or 300 nmole diquat in the right eyes, there was a dose-dependent rise in $O_2^{\cdot-}$ levels, 106-265 fold in the aqueous humor, 34-87 fold in the vitreous humor, 6-19 fold in the lens, and 43-88 fold in the retina as compared to 0.16 μM, 0.21 μM, 2.47 nmole/g and 5.56 nmole/g in tissues of the normal eyes, respectively. There were similar increases of $OH\cdot$ in the eye tissues, and of H_2O_2 in the aqueous humor and vitreous humor after diquat injection.

We propose that endogenous reductants of the eye tissues univalently reduce diquat to its free radical which spontaneously reacts with O_2 generating $O_2^{\cdot-}$ in excessive amounts, further giving rise to H_2O_2 and $OH\cdot$ triggering cataractogenesis.

KEY WORDS: Oxy radical assay, superoxide, hydroxyl radical, hydrogen peroxide, diquat-induced cataract, eye tissues.

INTRODUCTION

Cataract is a visual disorder in which the ocular lens loses its transparency and fails to refract the incident light so as to focus it on the retina for the normal visual acuity. Though the biochemical mechanism of the human senile cataract is not yet clearly understood, oxidative stress might be involved in the initiation of the pathology, and the reactive species of O_2 could be the triggering agents. However, there is no substantial experimental evidence supporting this hypothesis except for the reports showing marked increases of H_2O_2, one of the relatively stable reactive species of O_2 in the aqueous humor and vitreous humor of eyes in several experimental cataracts[1-3] and in the human senile cataract.[4-6]

In this communication, we describe experimental results showing the generation of oxy radicals in the eye tissues, and dose-dependant increases in the production of these radicals after administration of a bipyrydilium compound 1,1'-ethylene-2,2'-bipyridylium dibromide (Diquat), an O_2 free radical enhancer in the rabbit eye *in vivo*.

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MATERIALS AND METHODS

Chemicals

Diquat dibromide monohydrate (100% pure), was a gift from the Imperial Chemical Industries plc., London, U.K., disodium ethylenediaminetetraacetate (EDTA), trichloroacetic acid (TCA), 2,6-dichlorophenolindophenol sodium salt (Grade I), cytochrome *c* (Type IV) from horse heart, superoxide dismutase (SOD, purified from bovine erythrocyte, 3250 units/mg protein), horseradish peroxidase (Type VI), bovine liver catalase (2 × crystallized), salicylic acid and 2,3-dihydroxybenzoate were purchased from the Sigma Chemical Company. The other chemicals of certified grade were from the Fisher Scientific Company.

Test Animals

The animal investigations were conducted following the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Mount Sinai Medical Center, New York, and according to the resolution of the Association for Research in Vision and Ophthalmology, Inc., on the Use of Animals in Research. Healthy Dutch belted rabbits of either sex, 5- to 6-week-old, weighing 0.5 to 0.6 kg were used for the study. The animals with normal eyes were selected after examination of their eyes with a slit-lamp biomicroscope.

Intravitreal Injection of Diquat in Rabbit

Under general anesthesia and sterile conditions, a single dose of varying concentration (60–300 nmole) of diquat dibromide in 30 μl of 145 mM NaCl was injected intravitreally in the right eye of a rabbit. The left eye was kept as control by intravitreally injecting 30 μl of the vehicle. The eyes were examined daily with a slit-lamp biomicroscope to observe the lens changes. One week postinjection, rabbits were sacrificed and the eye tissues were taken for biochemical analyses.

Estimation of O_2^-

In the eye tissues, O_2^- was estimated by the technique described by Fridovich,[7] which is based on the mesurement of the SOD-inhibitable reduction of ferricytochrome *c* by the tissue extract at 550 nm. The assay system consisted of 0.025 mM ferricytochrome *c*, 50 mM $KH_2PO_4 \cdot K_2HPO_4$ buffer, pH 7.8, 0.1 mM EDTA, with or without 65 units of SOD and an appropriate aliquot of the aqueous humor, vitreous humor or homogenate of the lens or retina in a final volume of 1.3 ml incubated at 25°C for 15 min. At $\varepsilon_{550\,nm}$ ferricytochrome $c = 0.89 \times 10^4 \times M^{-1} \times cm^{-1}$ and $\varepsilon_{550\,nm}$ ferrocytochrome $c = 2.99 \times 10^4 \times M^{-1} \times cm^{-1}$. The concentration of O_2^- was calculated from the SOD-inhibitable cytochrome *c* reduced using $\Delta\varepsilon_{550\,nm} = 2.1 \times 10^4 \times M^{-1} \times cm^{-1}$.

Estimation of OH·

The technique described by Halliwell and Gutteridge,[8] was used to estimate OH· in the eye tissues. It is based on the measurement of 2,3-dihydroxybenzoate formed by

hydroxylation of salicylate by OH·. The reaction mixture consisted of 2 mM sodium salicylate, 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA and a suitable aliquot of aqueous humor or vitreous humor in a final volume of 1 ml or one preweighed lens or the whole retina from one eye in a final volume of 1.5 ml. The assay systems containing aqueous humor or vitreous humor were mixed, and those with the lens or retina were homogenized at 0–4°C, and incubated at 25°C for 30 min. The reactions were stopped by adding 40 μl of 10 M HCl and 0.25 g of NaCl/ml; 2,3-dihydroxybenzoate was extracted with chilled diethyl ether, and processed for colorimetric measurement at 510 nm. Pure 2,3-dihydroxybenzoate was used as a standard.

Estimation of H_2O_2

H_2O_2 in the aqueous humor and vitreous humor was estimated by the procedure described previously.[9-11] At 610 nm, pH 6.6, reduction of 2,6-dichlorophenolindophenol (oxidized blue form) by the ascorbic acid present in a suitable aliquot of the sample was followed by measurement of reoxidation of the reduced (leuco form) dye by H_2O_2 of the sample, in presence of horseradish peroxidase. Control reactions by adding catalase prior to the addition of peroxidase, accompanied the experimentals. From the equimolar stoichiometry of the reactions involved, H_2O_2 was determined using $\varepsilon_{610\,nm}$ oxidized dye $= 2.1 \times 10^4 \times M^{-1} \times cm^{-1}$.

Estimation of Diquat and Diquat Free Radical in the Eye Tissues

The 10,000 × g supernatant of the vitreous humor or that obtained from the homogenate of lens or retina, prepared in 50 mM phosphate buffer pH 7.8 were taken for the estimation of diquat and diquat free radical by absorption spectrophotometry. The spectra were scanned from 500 nm through 305 nm using a Beckman ratio recording spectrophotometer, Model DK-2A. All the spectra were corrected for absorption by tissue extracts of the normal eyes. Diquat and its free radical concentrations were calculated[12] using $\varepsilon_{310\,nm}$ diquat $= 1.92 \times 10^4 \times M^{-1} \times cm^{-1}$ and $\varepsilon_{375\,nm}$ diquat free radical $= 2.8 \times 10^4 \times M^{-1} \times cm^{-1}$.

TABLE I

Superoxide anion free radical ($O_2^{\cdot-}$) in the rabbit eye, one week after a single intravitreal injection of diquat in the right (Rt) eye *in vivo*

Diquat dosage nmole/eye[b]	$O_2^{\cdot-}$ · production[a] (SOD-inhibitable cytochrome c^{3+} reduced)			
	Aqueous humor [μM]	Vitreous humor [μM]	Lens (nmole/g)	Retina (nmole/g)
60/Rt	17.03 ± 3.83	7.15 ± 0.36	15.77 ± 1.82	237 ± 66
0/Lt	0.76 ± 0.10	0.92 ± 0.07	2.63 ± 0.48	14.96 ± 0.59
120/Rt	42.32 ± 5.75	18.33 ± 4.27	40.55 ± 4.44	488 ± 34
0/Lt	1.25 ± 0.22	1.06 ± 0.18	3.00 ± 0.18	33.51 ± 6.58
300/Rt	28.39 ± 4.44	16.06 ± 2.63	47.82 ± 4.99	473 ± 17
0/Lt	1.21 ± 0.22	1.03 ± 0.16	6.16 ± 0.93	68.30 ± 5.26

[a]Mean ± S.D., $n = 3$ eyes; [b]30 μl of diquat at varying concentration was injected intravitreally into the right (Rt) eye and the left (Lt) eye was injected similarly with the vehicle (145 mM NaCl). $P < 0.001$, control eye (Lt) vs. experimental (Rt), "t" test, one-tailed.

RESULTS

Superoxide Anion Free Radical in the Eye

The rates of $O_2^{\cdot-}$ formation over a period of 15 min were determined from the SOD-inhibitable reduction of ferricytochrome c^{3+} by the tissues or tissue extracts of the diquat-injected right eyes and vehicle-injected left eyes of rabbits. In normal eyes $O_2^{\cdot-}$ was $0.16 \pm 0.03\,\mu M$ (Mean $\pm$ S.D., $n = 6$ eyes) in the aqueous humor, $0.21 \pm 0.06\,\mu M$ in the vitreous humor, 2.47 ± 0.34 nmole/g wet wt in the lens, and 5.56 ± 0.76 nmole/g wet wt in the retina. The results given in Table I show that one week after a single intravitreal injection of diquat at a varying concentration of 60, 120 or 300 nmole per right eye, there were dose-dependent, significantly higher ($P < 0.001$) levels of $O_2^{\cdot-}$ 106, 265 and 177 fold in the aqueous humor, 34, 87 and 76 fold in the vitreous humor, 6, 16 and 19 fold in the lens and 43, 88 and 85 fold in the retina as compared to the $O_2^{\cdot-}$ levels in the tissues of the normal eyes, respectively. In the contralateral physiological saline-injected eyes of these rabbits, $O_2^{\cdot-}$ was higher 5–8 fold in the aqueous humor, about 5 fold in the vitreous humor, about 2 fold in the lens and 3–12 fold in the retina.

Hydroxyl Radical in the Eye

The rates of production of OH· were measured by the estimation of 2,3-dihydroxybenzoate formed in 30 min from the hydroxylation of salicylate by the tissues or tissue extracts of the diquat-injected right eyes and the vehicle-injected left eyes of rabbits. In normal eyes OH· was not detectable in the aqueous humor, it was $31.76 \pm 2.11\,\mu M$ (Mean $\pm$ S.D., $n = 6$ eyes) in the vitreous humor, 2.73 ± 0.38 nmole/g wet wt in the lens and 250 ± 29 nmole/g wet wt in the retina. The results given in Table II show that a week after a single dose of 60, 120 or 300 nmole diquat in the right eye, generation of OH· in the aqueous humor was significantly increased $P < 0.001$) 57, 121 and $202\,\mu M$ with the respective dosage. There was also a dose-dependent rise in the OH· 2 ($P < 0.05$), 3 ($P < 0.01$) and 7 fold ($P < 0.001$) in the vitreous humor, 6, 22 and 26 fold ($P < 0.001$) in the lens, and 2, 4 and 7 fold ($P < 0.001$) in the retina as compared to the normal levels, respectively. In the contralateral control eyes of these rabbits, OH· was detectable in the

TABLE II

Hydroxyl free radical (OH·) in the rabbit eye, one week after a single intravitreal injection of diquat *in vivo*

Diquat Dosage nmole/eye[b]	OH· production[a] (2,3-Dihydroxybenzoate formed)			
	Aqueous humor [μM]	Vitreous humor [μM]	Lens (nmole/g)	Retina (nmole/g)
60/Rt	56.95 ± 7.81	70.91 ± 6.17[c]	15.35 ± 1.68	473 ± 33
0/Lt	1.19 ± 0.31	42.84 ± 3.53	3.28 ± 0.47	260 ± 19
120/Rt	121 ± 20	100 ± 18[d]	59.43 ± 10.27	1051 ± 64
0/Lt	2.17 ± 0.46	53.04 ± 3.68	4.31 ± 1.03	287 ± 29
300/Rt	202 ± 27[d]	209 ± 19	69.74 ± 14.21	1788 ± 247
0/Lt	6.96 ± 1.27	73.05 ± 8.40	8.32 ± 2.09	364 ± 15

[a]Mean ± S.D., $n = 3$ eyes; [b]30 μl of diquat at varying concentration was injected intravitreally into the right (Rt) eye and the left (Lt) eye was injected similarly with the vehicle (145 mM NaCl). [c]$P < 0.05$, [d]$P < 0.01$ and the rest $P < 0.001$, control eye (Lt) vs. experimental (Rt), "t" test, one-tailed.

TABLE III

H_2O_2 in the aqueous humor and vitreous humor of rabbit, one week after a single intravitreal injection of diquat in the right (Rt) eye *in vivo*

Diquat Dosage nmole/eye[a]	H_2O_2 Concentration [μM], Mean ± S.D., n = 3 eyes	
	Aqueous humor	Vitreous humor
60/Rt	56 ± 8	46 ± 7
0/Lt	37 ± 2	25 ± 4
120/Rt	99 ± 15	89 ± 7
0/Lt	37 ± 7	25 ± 3
300/Rt	118 ± 19	77 ± 5
0/Lt	39 ± 6	29 ± 6

[a] 30 μl of diquat at varying concentration was injected intravitreally into the right (Rt) eye and the left (Lt) eye was injected similarly with the vehicle (145 mM NaCl). $P < 0.001$, control eye (Lt) vs. experimental (Rt), "t" test, one-tailed.

aqueous humor, was about 2 fold in the vitreous humor, 1–3 fold in the lens and was unaltered in the retina as compared to the normal levels respectively.

Hydrogen Peroxide in the aqueous humor and vitreous humor

H_2O_2 levels in the aqueous humor and vitreous humor were estimated, one week after a single intravitreal injection of a varying dose of diquat in the right eyes of rabbits. In normal eyes H_2O_2 was 32 ± 6 μM (Mean ± S.D., n = 6 eyes) in the aqueous humor, and 18 ± 3 μM in the vitreous humor. From the results given in Table III it is seen that with a dose of 60, 120 or 300 nmole diquat, H_2O_2 was significantly higher ($P < 0.001$) than normal levels by 2, 3 and 4 fold in the aqueous humor and 3, 5 and 4 fold in the vitreous humor respectively. In the contralateral physiological saline-injected eyes, H_2O_2 levels in the aqueous humor and vitreous humor were close to normal.

Diquat and Diquat Free Radical in the Eye

A week after a single intravitreal injection of diquat at a varying concentration in the

TABLE IV

Diquat (DQ) and its free radical (DQ·) in the rabbit eye one week after a single intravitreal injection of the compound in the right (Rt) eye *in vivo*

DQ Dosage nmole/eye[a]	DQ and DQ· concentrations in the eye (% of the injected amount per eye, n = 3)					
	Lens		Vitreous humor		Retina	
	DQ	DQ·	DQ	DQ·	DQ	DQ·
60/Rt	6.0	2.1	10.0	1.0	22.6	7.9
0/Lt	0.5	0.2	0.0	0.0	1.1	0.1
120/Rt	6.3	2.3	12.7	1.0	27.6	6.9
0/Lt	0.8	0.4	0.0	0.0	0.8	0.2
300/Rt	9.3	2.3	12.0	1.0	—	—
0/Lt	0.9	0.6	0.0	0.0	0.4	0.1

[a] 30 μl of diquat at varying concentration was injected intravitreally into the right (Rt) eye and the left (Lt) eye was injected similarly with the vehicle (145 mM NaCl).

right eyes of rabbits, the distribution of this compound and its free radical in the eye tissues were measured spectrophotometrically. The data given in Table IV show that at 60, 120 or 300 nmole dosage of diquat, its levels expressed as percent of the administered dosage were 6–9 in the lens and 10–13 in the vitreous humor. It was 23–28% in the retina after 60 or 120 nmole intravitreal diquat injection. At a higher dose of diquat, the retina was severely degenerated and was not available for analysis. The diquat free radical produced after 60, 120 or 300 nmole diquat was 2% of the injected doses of diquat in the lens, 1% in the vitreous humor and 7–8% in the retina. A trace amount of diquat was also detectable in the control left eyes, 0.5–0.9% diquat and 0.2–0.6% diquat free radical in the lens, about 1% diquat and 0.15% diquat free radical in the retina, and no diquat or its free radical was detected in the vitreous humor.

DISCUSSION

Oxygen free radicals being the extremely short-lived reactive species, it is not possible to measure their cellular steady state levels, though there is no doubt that they are generated in the biological tissues.[13] The evidence presented here demonstrates that productions of $O_2^{\cdot-}$, $OH\cdot$ and H_2O_2 in the eye tissues were enhanced by the redox active xenobiotic diquat in rabbit eyes *in vivo*, and it was possible to estimate these reactive O_2 species in the eye tissues by using the indirect spectrophotometric techniques.[7–11]

The rates of production of the reactive species of O_2 were significantly higher in the eye tissues after a single intravitreal dose of diquat (60–300 nmole) in rabbit eyes *in vivo*, than in the physiological saline-injected contralateral control eyes. As compared to the levels of O_2 free radicals and H_2O_2 in normal eye tissues, increases in the concentrations of $O_2^{\cdot-}$, $OH\cdot$ and H_2O_2 in the aqueous humor and vitreous humor, and $O_2^{\cdot-}$ and $OH\cdot$ in the lens and retina of the eyes after 60 or 120 nmole of intravitreal diquat, were directly proportional to the injected dose. However, their rates of production in the eye tissues after 300 nmole diquat injection, either plateaued showing no further rise or a slight fall. It is likely that diquat, at this level, saturated the endogenous redox systems and though in excess, could not be cyclically reduced and oxidized to generate more oxy radicals. It is important to note that the severity of diquat-induced cataract was also directly related to the concentrations of the oxy radicals produced. As observed with a slit-lamp biomicroscope, no cataract was detected within a week after injection of 60 nmole diquat, a dose of 120 nmole diquat induced early changes of cataract (grade 1), and with 300 nmole diquat cataract (grade 1) was visible within 1–3 days, which advanced to grade 2 in 3–5 days.

After a single intravitreal injection, diquat was detected in the retina, lens and vitreous humor. Diquat free radical was also present in these tissues. Of the injected dose of diquat, the highest amount 22–28% was in the retina, 10–13% in the vitreous humor and 6–9% in the lens a week postinjection. The production of diquat free radical was also highest in the retina about 8% of the injected dose of diquat, 1–2% in the lens and 1% in the vitreous humor. The distribution of diquat free radical in eye tissues indicates that its production is related to the availability of the endogenous reductants which are highest in the retina and least in the vitreous humor. By injecting ^{14}C-diquat intraperitoneally in rats, it has been shown that diquat reaches the eye tissues in an hour after the injection.[14]

It has been observed that the free radical produced by one electron reduction of the redox active bipyridylium compound, diquat[15] or paraquat[16] reacts with O_2, generating O_2^- and the original compound. We propose that by cyclic reduction and oxidation, diquat might divert the flow of electrons from the endogenous redox systems of the eye to O_2 so as to produce O_2^- in excessive amounts further generating H_2O_2 and OH· which might be the responsible oxidants triggering cataractogenesis.

The indirect spectrophotometric techniques to measure O_2^- in the biological tissues as described by Fridovich,[7] OH· as described by Halliwell and Gutteridge,[8] and H_2O_2 as described by Mapson,[10] Pirie[11] and us[9] are sensitive, reproducible and suitable for the measurements of O_2^-, OH· and H_2O_2 produced in the eye tissues by diquat *in vivo*.

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