ELSEVIER

Biochimica et Biophysica Acta 1285 (1996) 21–28

BBA

Crosslinking of aminophospholipids in cellular membranes of lens by oxidative stress in vitro

Durga K. Bhuyan, Ravidatt W.P. Master, Kailash C. Bhuyan *

Membrane Biochemistry Laboratory, The Edward S. Harkness Eye Institute, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, NY 10032, USA

Received 25 March 1996; revised 22 July 1996; accepted 24 July 1996

Abstract

We have previously demonstrated by TLC an additional phospholipid spot between phosphatidylethanolamine (PE) and phosphatidylserine (PS) in human cataract. This was further identified as a fluorescent Schiff-base conjugate resulting from crosslinking of reactive carbonyl groups of malondialdehyde (MDA) with the primary amino groups of membrane phospholipids. Evidence presented here shows that such an adduct could be formed in rabbit lens subjected to oxidative stress in vitro. TLC analysis of a lipid extract of a crude membrane fraction obtained from the lens homogenate incubated with 1 mM H_2O_2, *tert*-butyl hydroperoxide (TBHP) or MDA for 1–6 h at 25°C, showed that the oxidants and MDA produced time-dependent crosslinking of aminophospholipids. Under identical conditions of incubation with TBHP or MDA, development of the Schiff-base lipid fluorochrome in lens with peak emission at 470 nm when excited at 360 nm also showed a time-dependent increase. The PE · MDA · PS produced in cellular membranes of the lenses cultured for 3 h in Krebs–Ringer medium was 151 nmol/μmol PE, and addition of 1 mM H_2O_2, TBHP or MDA, increased it to 881, 610 and 375 nmol/μmol PE, respectively. Adduct was also formed when authentic samples of PE and PS were reacted with pure MDA. From the results it is clear that oxidants viz., H_2O_2 and TBHP, or MDA were effective in promoting crosslinking of lens membrane aminophospholipids by Schiff-base conjugation of primary amino groups with the carbonyl groups of the aldehyde, a breakdown product of lipid peroxides.

Keywords: Aminophospholipid · MDA; Cataract; Conjugated diene; Hydrogen peroxide; Lipid peroxide; Malondialdehyde; Oxidative stress; (Lens cellular membrane)

1. Introduction

The mechanism which initiates the progression of cataract is a subject of continuing investigation, and is still far from clear. In the majority of cataracts, oxidative damage to the biomolecules of lens is common. The accumulation of high molecular weight intracellular protein aggregates has been repeatedly observed in cataract, due mainly to formation of disulfide and to a lesser extent non-disulfide crosslinks [1–3]. However, the cellular plasma membrane is the primary site of oxidant-initiated injury due to its content of oxidizable lipid and protein, and its proximity to both intra- and extracellular sources of reactive species of oxygen [4,5]. Lipids containing polyunsaturated fatty acyl side chains are more prone to oxidation than membrane-associated proteins, and are critical to the maintenance of their structural and functional integrity. Lipid peroxide can initiate de-

* Corresponding author. Fax: +1 (212) 3052768; e-mail: kcb4@columbia.edu.

PII S0005-2736(96)00142-3

struction of cysteinyl-SH groups and methionyl residues of proteins [6] and cause altered membrane functions in lens [7]. Oxygen free radical- and hydrogen peroxide-initiated peroxidation of membrane lipids has been considered a mechanism for cellular damage [4,5,8]. Formation of lipid hydroperoxides is accompanied by their decomposition into a variety of reactive aldehydes [9], among which malondialdehyde (MDA) and 4-hydroxy-2,3-transnonenal (4-HNE) are cytotoxic. There is increasing evidence that aldehydes produced in membranes could be causally related to many diseases resulting from oxidative stress [4,10–12]. We and others have observed enhanced formation of MDA, a thiobarbituric acid (TBA)-reactive material in maturity-onset human cataract [13–16] and various experimental cataracts [15,16]. Subsequently, a mechanism of cataract formation was proposed which involves aerobic free radical-initiated peroxidation of membrane lipids, resulting in the formation of MDA as one of the aldehydic breakdown products that could crosslink with primary amino groups of proteins and amino groups of phospholipids and nucleic acids [15]. Formation of aminophospholipid · MDA, a Schiff-base conjugate, was originally noted in tissues of animals raised on vitamin E-deficient diets and in aged animals [4,17]. As a result of peroxidative damage in sickled erythrocytes, a similar product was found [18], and we have shown the presence of such an adduct in maturity-onset human cataract [19]. On thin-layer chromatographic analysis, a novel compound was observed between phosphatidylethanolamine (PE) and phosphatidylserine (PS), among the major phospholipids of the lens. In normal lenses, this PE · MDA · PS adduct was considerably less than in cataractous lenses [19–21]. In this communication, we present data showing Schiff-base crosslinking of aminophospholipids with MDA by reactions in lens in vitro initiated by oxidants such as H_2O_2 or the organic peroxide, *tert*-butyl hydroperoxide (TBHP).

2. Materials and methods

2.1. Chemicals

1,1',3,3'-Tetraethoxypropane was from K and K Laboratories (Plainview, NY); 2,6-di(*tert*-butyl)-4-methylphenol (BHT), chloroform and methanol of analytical grade were from Aldrich (Milwaukee, WI); TBA, ninhydrin (0.2%, w/v, in ethanol) and molybdenum blue (1.3% molybdenum oxide in 4.2 M H_2SO_4) spray reagents, xanthine (X), bovine milk xanthine oxidase (XOD, crystalline suspension), TBHP (70% aqueous solution), hydrogen peroxide (H_2O_2, 30%, w/w) and 3-amino-1H-1,2,4-triazole (3-aminotriazole) were purchased from Sigma (St. Louis, MO). Reference phospholipids, PE, PS, phosphatidylcholine (PC) and sphingomyelin (SP), were obtained from Supelco (Bellefonte, PA) and all other chemicals of analytical grade were from Fisher (Springfield, NJ). The silica gel HL with organic binder thin-layer chromatography (TLC) plates 20 × 20 cm, 250 μm thick were from Analtech (Newark, DE).

2.2. Test animals

Healthy 5–6-week-old pigmented rabbits of either sex were killed by CO_2 euthanasia, and normal fresh lenses were used for in vitro experiments. Individual fresh lens weight varied from 100 to 120 mg. The animal investigation was conducted following the Guide for the Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 86-23, Office of Science and Health Reports, DRR/NIH, Bethesda, MD, and Recommendations from the Declaration of Helsinki.

2.3. Extraction and isolation of total lipids, and quantification of phospholipids

The lens homogenate prepared in 2 ml of 0.15 M NaCl containing 10 mM Tris-HCl buffer (pH 7.4) and 23 μM BHT (a lipid antioxidant) was centrifuged at 45900 × *g* for 20 min, and the crude membrane pellet [22] was extracted [19,23] with $CHCl_3/CH_3OH$ (2:1, v/v), and $HClO_4$ at 1% final concentration was added to precipitate the proteins and to facilitate separation of the phases. The $CHCl_3$ phase was dried at 37°C under nitrogen and further purified by washing with $CHCl_3/CH_3OH/H_2O$ (2:1:1, v/v) to remove flavins and water-soluble contaminants. The final $CHCl_3$ phase was dried under nitrogen, dissolved in 15 μl chloroform, and a 10 μl aliquot was spotted on TLC plate. The solvent

system for TLC separation of the phospholipids was $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50:25:7:3, v/v). After development and drying, the TLC plate was briefly exposed to iodine vapor in a glass chamber. The phospholipids were seen as dark-brown spots against a pale yellow background. Aminophospholipids were visualized as violet-purple spots by spraying the TLC plates, after the run, with 0.2 percent ninhydrin in ethanol, followed by heating at 70–80°C for 2 h or longer. The phosphate-containing spots were visualized by the molybdenum spray reagent. For a permanent record, the TLC plates, after development, were sprayed with 40% H_2SO_4 and heated at 105–110°C for 2 h or longer. Charred spots against a white background were obtained as residues of the phospholipid components. Authentic samples of phospholipids were used as standards.

The boundaries of the separated phospholipid components, as visualized by brief exposure to iodine vapor, were carefully marked, and the silica was quantitatively scraped into borosilicate tubes. To each tube, 0.4 ml 70% perchloric acid was added, and the contents of the tubes were individually digested on a flame till white silica gel turned brownish-black and ultimately became clear. Each sample was diluted to 2.0 ml with water and for the estimation of inorganic phosphorus, an equal volume of the reagent containing 1.2 N H_2SO_4, 0.5% ammonium molybdate and 2% ascorbic acid [24] was added, the mixture was gently vortexed and incubated at 37°C for 2 h or longer in the dark. The color intensities were measured at 800 nm. The concentration of individual phospholipids was calculated from the inorganic phosphorus content of each spot. Appropriate blanks were included at all stages of the experiment. The R_f values were determined by running authentic standards, and the recoveries estimated by applying known amounts of phospholipid and measuring the inorganic phosphorus after the chromatographic procedure.

2.4. Lens organ-culture system

For quantitative measurement of the PE · MDA · PS adduct under conditions of oxidative stress or exposure to MDA, normal lenses were cultured in modified Krebs–Ringer bicarbonate (KRB) medium composed of 143 mM Na^+, 5.1 mM K^+, 1.5 mM Ca^{2+}, 1.0 mM Mg^{2+}, 27 mM HCO_3^-, 125 mM Cl^-, 0.47 mM HPO_4^{2-}, 0.11 mM $H_2PO_4^-$ and 5 mM glucose. Each lens was placed in a glass vial containing KRB medium with or without the oxygen free radical generating system (X, 2.5 mM; XOD, 0.05 IU/ml; $FeCl_3$, 0.12 mM; ADP, 0.3 mM), H_2O_2, TBHP or MDA in a final volume of 8 ml (pH 7.4) and gas phase 95% O_2 and 5% CO_2. Incubation was done at 37°C for 3 h in a water bath with shaking at 72 oscillations per min. When XOD was present, it was always introduced after all other additions to start the reaction. When H_2O_2 was used to produce oxidative stress, lenses were pre-incubated with 10 mM 3-aminotriazole to inhibit the endogenous lens catalase. Appropriate controls were run for all experiments. At the end of incubation, each lens was rinsed with 0.15 M NaCl containing 23 μM BHT, homogenized in 2 ml saline containing BHT, and the membrane pellet was processed for extraction of total lipids, which were analyzed for phospholipids by TLC [19] as described earlier in the text. MDA used in these experiments was freshly synthesized by hydrolysis of 1,1',3,3'-tetraethoxypropane with 0.1 N HCl for 24 h at 25°C.

2.5. Incubation of lens homogenate

For kinetic studies, lens homogenate (20%, w/v) was prepared in 0.15 M NaCl containing 10 mM Tris-HCl buffer (pH 7.4). Homogenate, 1.75 ml, was incubated in presence of 1 mM H_2O_2, TBHP or MDA in a final volume of 2.0 ml (pH 7.2) at 25°C, with shaking at 72 oscillations per min, for 0, 1, 3 or 6 h. The incubation mixture was centrifuged at 45900 × g for 30 min to obtain a crude membrane pellet [22] which was analyzed for phospholipids as described earlier.

2.6. Estimation of lipid fluorochrome

The purified chloroform phase containing lens membrane lipids, which were free of flavins and aqueous contaminants, was used for measurement of fluorescence spectra with peak emission at 470 nm when excited at 360 nm [4,25]. Quinine sulfate (1 μM in 0.1 N H_2SO_4) was used as fluorescence standard.

2.7. Measurement of conjugated dienes

For estimation of conjugated dienes [26], the lipid extracts in chloroform were dried under nitrogen, dissolved in methanol, and absorbance was measured at 233 nm. Conjugated diene was calculated from ε (233 nm, conjugated dienes) = $2.8 \cdot 10^4$ M^{-1} cm^{-1} and expressed as nmol/mg total phospholipid.

2.8. Estimation of MDA (TBA-reactive material)

Pink, trimethine condensation product, formed by the reaction of 1 mol MDA with 2 mol TBA at 100°C in acidic condition [27], was measured in an aliquot of protein-free trichloroacetic acid supernatant obtained from lens [15], ε (533 nm, MDA) = $1.5 \cdot 10^5$ M^{-1} cm^{-1}. MDA was expressed as nmol/g lens.

3. Results

3.1. Phospholipid composition of the cellular membranes of lens

A typical TLC profile of phospholipids of normal rabbit lens is shown in Fig. 1, lane 1, and lane 5 shows separation of a mixture of phospholipid standards. The individual phospholipid spots separated on TLC were quantitated by measuring phosphorus content, and the results are summarized in Table 1 which shows that the four major phospholipids were present in the cellular membranes of the normal rabbit lens. The R_f values for SP, PC, PS and PE of authentic samples in increasing order were 0.14, 0.32, 0.57 and 0.75, respectively, and the recoveries were 78, 62, 95 and 100 percent, respectively.

Table 1
Phospholipid composition of normal rabbit lens

	Phospholipid concentration (μg/g wet wt.) [a]
Phosphatidylethanolamine (PE)	152 ± 17
Aminophospholipid adduct	5 ± 3
Phosphatidylserine (PS)	176 ± 29
Phosphatidylcholine (PC)	183 ± 21
Sphingomyelin (SP)	139 ± 26

[a] Mean ± S.D., n = 4 lenses.

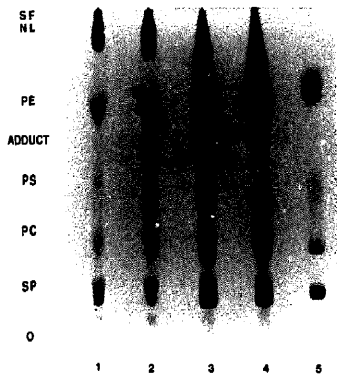

Fig. 1. TLC profiles of lipid extracts of crude membrane pellets obtained from rabbit lens homogenate incubated in absence (control), or in presence of 1 mM H_2O_2, TBHP or MDA for 3 h at 25°C. Lane 1, control; Lane 2, with 1 mM H_2O_2; Lane 3, with 1 mM TBHP; Lane 4, with 1 mM MDA and Lane 5, Standard phospholipids, 10 μl mixture (2 mg each of PE, PS, PC and SP/ml $CHCl_3$). NL, neutral lipids.

A novel additional phospholipid component having a R_f value (0.66) intermediate between those of PE and PS, which was also observed in cataractous human lens, and was characterized as PE · MDA · PS

Table 2
Oxidative stress-induced lipid peroxidation of rabbit lens in vitro

Additions to KRB medium	Malondialdehyde in lens (TBA-reactive material)	
	nmol/g wet wt.	% control
None (control)	1.17 ± 0.10 (10) [a]	100
Xanthine (X), 2.5 mM	1.63 ± 0.18 (4)	139
XOD, 0.05 IU/ml	1.33 ± 0.11 (4)	114
X + XOD	1.93 ± 0.28 (4)	165 [b]
$FeCl_3$, 0.12 mM	1.41 ± 0.12 (4)	121
ADP, 0.3 mM	1.22 ± 0.14 (4)	104
Fe^{3+} · ADP	1.49 ± 0.15 (6)	127
X + XOD + Fe^{3+} · ADP	4.08 ± 0.23 (8)	349 [b]

Normal rabbit lenses were incubated at 37°C with shaking for 3 h in vials containing 8 ml of KRB medium (pH 7.4) and gas phase of 95% O_2 and 5% CO_2. Additions to the incubation system were as shown in the table. When XOD was used, it was always introduced after all other additions to start the reaction. Lenses were rinsed subsequently with 0.15 M NaCl containing 23 μM BHT, and MDA was estimated.

[a] Mean ± S.D., (n) = number of lenses. [b] $P < 0.001$; unpaired, one-tailed statistical t-test of experimental vs. control lenses; the differences of others are not significant.

adduct [19] was either present in trace amounts or absent in normal rabbit lens.

3.2. Lipid peroxidation in lens initiated by reactive species of oxygen

As determined by the TBA test (refer to Table 2), MDA in control lenses was 1.17 nmol/g. Compared to this value, in the presence of X-XOD-Fe^{3+}-ADP which generates reactive species of oxygen, MDA was higher ($P < 0.001$) by 249%; in presence of xanthine alone it was 39% higher, and with X-XOD (without Fe^{3+}-ADP) it was 65% higher.

3.3. Crosslinking of aminophospholipids in cellular membranes of lens

Formation of PE · MDA · PS adduct in lenses incubated in KRB medium, supplemented with the X-XOD-Fe^{3+}-ADP system or with varying concentrations of oxidants such as H_2O_2 or TBHP or with pure MDA, was estimated by quantitative TLC. The results summarized in Table 3 show that formation of PE · MDA · PS in lens membranes was dependent on the concentration of oxidants (H_2O_2 or TBHP) or MDA itself. The level of adduct was about twofold higher in lenses incubated with the complete system, generating reactive species of oxygen (X-XOD-Fe^{3+}-ADP), compared with the amount of adduct, 151 nmol/μmol PE, in control lenses incubated in KRB medium alone. The formation of adduct in the presence of oxidants (H_2O_2 or TBHP) or MDA itself was concentration-dependent. In the presence of 1 mM H_2O_2, TBHP or MDA, its level was significantly higher by 6-, 4- and 3-fold than the control value, respectively. This indicated that oxidative stress-initiated lipid peroxidation in lens generated MDA that reacted with the amino groups of PE and PS forming a Schiff-base conjugate, which was identical to that formed with pure MDA.

Table 3
Crosslinking of aminophospholipids of rabbit lens under oxidative stress, and in presence of MDA in vitro

Additions to KRB medium		PE · MDA · PS of lens [a]	
		nmol/μmol PE	%
None (control)		151 ± 13	100
X · XOD · Fe^{3+} · ADP		265 ± 18	175 [b]
H_2O_2	0.1 mM	191 ± 8	126
	1.0 mM	881 ± 24	583 [c]
	10.0 mM	939 ± 45	622 [c]
TBHP	0.1 mM	429 ± 27	284 [b]
	1.0 mM	610 ± 30	404 [c]
MDA	0.1 mM	269 ± 12	178 [b]
	1.0 mM	375 ± 17	248 [c]
	10.0 mM	646 ± 29	428 [c]

Normal lenses of rabbits were incubated in KRB medium with additions as indicated in the table. Conditions were as described in the caption to Table 2. After incubation, the lenses were analyzed for aminophospholipid adduct (PE · MDA · PS) by TLC.
[a] Each lens was pre-incubated with 10 mM 3-aminotriazole at 37°C for 1 h prior to the addition of H_2O_2. The values are expressed as mean ± S.D., $n = 3$ lenses. [b] $P < 0.01$ and [c] $P < 0.001$; unpaired, one-tailed statistical t-test of experimental vs. control lenses.

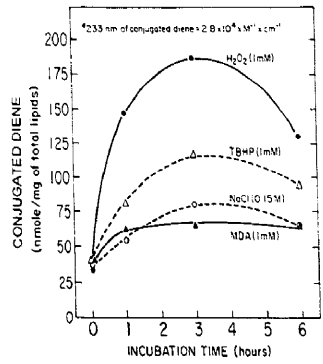

Fig. 2. Formation of conjugated dienes in cellular membranes of lens as a function of time under oxidative stress. Lipid extracts from membrane pellets were obtained from lens homogenates incubated at 25°C with 0.15 M NaCl (control) (○---○); 1 mM H_2O_2 (●———●); 1 mM TBHP (△---△) or 1 mM MDA (▲———▲). The data are averages of duplicate experiments. Detailed procedures are given in Section 2 in the text.

To observe the time-dependent reactions of oxidants or MDA, promoting lipid peroxidation and PE · MDA · PS formation in cellular membranes of lens, a homogenate of lens was incubated with 1 mM H_2O_2, TBHP or MDA at 25°C for 1 h, 3 h and 6 h. Control samples without addition of oxidants or MDA accompanied the test. At the end of incubation, total lipid extracts, isolated from the crude membrane pellets obtained from the various homogenate mixtures, were analyzed. As seen from the data represented graphically in Fig. 2, formation of conjugated dienes in lens, measured spectrophotometrically at

233 nm, increased with time up to 3 h in presence of oxidants. Production of conjugated dienes at 3 h was higher than control values by 134% and 43% with 1 mM H_2O_2 or TBHP, respectively. MDA, a breakdown product of lipid peroxide, did not produce any conjugated dienes.

From the results shown in Fig. 3, it is evident that accumulation of PE · MDA · PS adduct in cellular membranes of lens after reaction with 1 mM H_2O_2 or TBHP was a function of time up to 6 h. Formation of adduct also occurred in presence of 1 mM MDA, increasing with time to 3 h, and plateaued thereafter, perhaps due to the limited availability of PE and PS in the sample of lens homogenate.

Fig. 4 shows relative fluorescence at 470 nm peak emission when excited at 360 nm of the characteristic Schiff-base lipid fluorochrome produced in lens membrane lipids after incubation of lens homogenate with oxidants or MDA. Lipid fluorochrome increased significantly in presence of 1 mM MDA, and its rise was a function of time over the 6 h period, whereas,

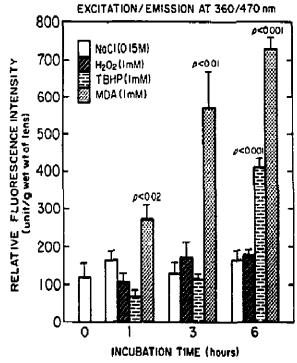

Fig. 4. Formation of Schiff-base lipid fluorochrome in the cellular membranes of lens under oxidative stress as a function of time. The total lipids, isolated from membrane pellets, were obtained from rabbit lens homogenates incubated with H_2O_2, TBHP or MDA at 25°C. The relative fluorescence of a quinine sulfate standard (1 μM in 0.1 N H_2SO_4) was 486 units at emission 470 nm on excitation at 360 nm.

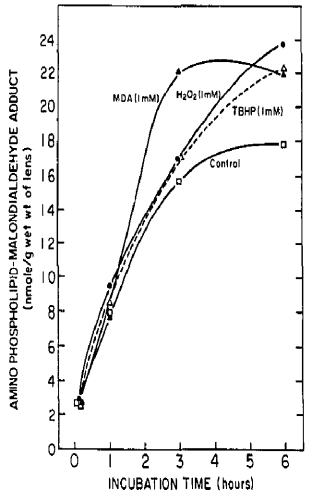

Fig. 3. Formation of aminophospholipid·MDA adduct in cellular membranes of lens under oxidative stress as a function of time. The adduct was isolated and quantified in lipid extracts of membrane pellets obtained from lens homogenates incubated at 25°C with no addition (control) (□———□); with 1 mM H_2O_2 (●———●); 1 mM TBHP (△---△) or 1 mM MDA (▲———▲). The data are averages of duplicate experiments. Detailed procedures are given in Section 2 in the text.

in presence of 1 mM TBHP, it increased significantly only after a 6 h incubation. In the presence of 1 mM H_2O_2, however, no rise in lipid fluorochrome could be detected up to 6 h. This is probably due to the drastic quenching effect of H_2O_2 on fluorescence of PE · MDA · PS, a Schiff-base conjugate itself.

4. Discussion

The data presented demonstrate that four major phospholipid components, PE, PS, PC and SP, were present in cellular membranes of young rabbit lens. Compared to the concentrations of these phospholipid constituents reported in adult rabbit lens [28–30], levels of PE, PS and PC were considerably higher and SP was lower in young lenses. Similar decreases in phospholipid concentration were found in human lenses on aging [31,32].

Aminophospholipids are important structural constituents of cellular membranes of lens, and most likely to be susceptible to oxidative damage by reactive species of oxygen or hydrogen peroxide. Administration of 3-aminotriazole [15,33,34] or 1,1'-ethylene-2,2'-bipyridylium dibromide (Diquat) [35,36] in rabbits has revealed that excessively produced ocular

H_2O_2, O_2^- and $^{\cdot}OH$ in vivo, due to impairment of antioxidant enzymes, were agents triggering cataractogenesis. Involvement of toxic metabolites of oxygen in maturity-onset human cataract was indicated by elevated levels of H_2O_2 in the aqueous humor [14,15,37], vitreous humor [13–15] and the lens [37,38].

In cellular membranes of lenses exposed to oxidants such as H_2O_2 or TBHP in vitro, there was a time-dependent promotion of lipid peroxidation, indicated by the formation of lipid conjugated dienes and MDA. Crosslinking of aminophospholipids by Schiff-base conjugation with carbonyl groups of MDA also occurred, as evidenced by the formation of PE · MDA · PS adduct, and rise in lipid fluorochrome with fluorescence characteristic of a Schiff-base conjugate. Formation of such products by these reactions was a function of concentration of the oxidants. The aminophospholipid · MDA adduct produced under oxidative stress was identical to that produced in lens after incubation with pure MDA. We have identified the same product in human maturity-onset cataract [19], characterized it as a PE · MDA · PS adduct by Raman spectroscopy [39] and demonstrated its preponderance in cataracts compared with normal lens of the human [19].

Another highly reactive aldehyde arising in tissues from peroxidation of lipids is 4-HNE, which elicits potent cytotoxic effects [9–11] due in part to Michael addition-type reactions with amino acid residues of proteins, generating protein carbonyls [9–12]. Another interaction of the aldehydic carbonyl of 4-HNE is with the primary amino group of PE and/or PS, which produces a lipid fluorochrome with emission maximum at 430 nm when excited at 360 nm [40]. A fluorescent proteinaceous product can arise on incubation of low density lipoprotein with 4-HNE [9,41]. Some of the fluorescence noted (Fig. 4) could be due to reaction of 4-HNE, although the presence of this compound in the lens has yet to be demonstrated.

Based on the evidence presented, it is concluded that formation of PE · MDA · PS in lens cell membranes under oxidative stress is very likely to be one of the early events occurring in the precataractous lens. The data provide suggestive evidence of its involvement in the mechanism of cataractogenesis that was proposed previously [15,33,34].

Acknowledgements

This study was supported by a grant, EY03012, from the National Eye Institute, NIH, and an unrestricted grant from Research to Prevent Blindness, Inc., New York. The authors gratefully thank Dr. Harold L. Kern for pre-reviewing the manuscript with constructive comments.

References

[1] Augusteyn, R.C. (1981) Protein modification in cataract: Possible oxidative mechanisms, in Mechanisms of Cataract Formation in the Human Lens (Duncan, G., ed.), pp. 72–115, Academic Press, New York.
[2] Spector, A. (1984) Oxidation and cataract, in Ciba Foundation Symposium 106, Human cataract formation (Nugent, J. and Whelan, J., eds.), pp. 48–64, Pitman, London, UK.
[3] Harding, J.J. (1991) Cataract. Biochemistry, Epidemiology and Pharmacology, Chapman and Hall, London.
[4] Tappel, A.L. (1975) in Pathobiology of Cell Membranes, (Trump, B.F. and Arstila, A.U., eds.), Vol. 1, pp. 145–170, Academic Press, New York.
[5] Freeman, B.A. and Crapo, J.D. (1982) Lab. Invest. 47, 412–426.
[6] Lewis, S.E. and Wills, E.D. (1962) Biochem. Pharmacol. 11, 901–912.
[7] Bhuyan, K.C., Bhuyan, D.K., Kuck, J.F.R., Jr., Kuck, K.D. and Kern, H.L. (1982/1983) Curr. Eye Res. 2, 597–606.
[8] O'Brien, P.J. (1987) in Autoxidation of Unsaturated Lipids (Chan, H.W.S., ed.), pp. 233–280, Academic Press, New York.
[9] Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) Free Radic. Biol. Med. 11, 81–128.
[10] Grune, T., Siems, W.G., Zollner, H. and Esterbauer, H. (1994) Cancer Res. 54, 5231–5235.
[11] Witz, G. (1989) Free Radic. Biol. Med. 7, 333–349.
[12] Uchida, K. and Stadtman, E.R. (1994) Methods Enzymol. 233, (Part C) 371–380.
[13] Bhuyan, K.C., Bhuyan, D.K. and Podos, S.M. (1981) IRCS Med. Sci. 9, 126–127.
[14] Bhuyan, K.C., Bhuyan, D.K. and Podos, S.M. (1986) Life Sci. 38, 1463–1471.
[15] Bhuyan, K.C. and Bhuyan, D.K. (1984) Curr. Eye Res. 3, 67–81.
[16] Varma, S.D., Chand, D., Sharma, Y.R., Kuck, Jr., J.F.R. and Richards, R.D. (1984) Curr. Eye Res. 3, 35–57.
[17] Reddy, K., Fletcher, B. and Tappel, A.L. (1973) J. Nutr. 103, 908–915.
[18] Jain, S.K. and Shohet, S.B. (1984) Blood 63, 362–367.
[19] Bhuyan, K.C., Master, R.W.P., Coles, R.S. and Bhuyan, D.K. (1986) Mech. Ageing Dev. 34, 289–296.

[20] Borchman, D., Yappert, M.C., Rubini, R.Q. and Paterson, C.A. (1989) Curr. Eye Res. 8, 939–946.
[21] Babizhayev, M.A. (1989) Acta Ophthalmol. 67, 281–287.
[22] Alcala, J., Valentine, J. and Maisel, H. (1980) Exp. Eye Res. 30, 659–677.
[23] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
[24] Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758.
[25] Bidlack, W.R. and Tappel, A.L. (1973) Lipids 8, 203–207.
[26] Recknagel, R.O. and Ghoshal, A.K. (1966) Lab. Invest. 15, 132–148.
[27] Dahle, L.K., Hill, E.G. and Holman, R.T. (1962) Arch. Biochem. Biophys. 98, 253–261.
[28] Broekhuyse, R.M. (1971) Biochim. Biophys. Acta 218, 546–548.
[29] Zelenka, P.S. (1984) Curr. Eye Res. 3, 1337–1359.
[30] Greiner, J.V., Auerbach, D.B., Leahy, C.D. and Glonek, T. (1994) Invest. Ophthalmol. Vis. Sci. 35, 3739–3746.
[31] Li, L.-K., So, L. and Spector, A. (1987) Biochim. Biophys. Acta 917, 112–120.
[32] Borchman, D., Byrdwell, W.C. and Yappert, M.C. (1994) Invest. Ophthalmol. Vis. Sci. 35, 3938–3942.
[33] Bhuyan, K.C. and Bhuyan, D.K. (1977) Biochim. Biophys. Acta 497, 641–651.
[34] Bhuyan, K.C. and Bhuyan, D.K. (1978) Biochim. Biophys. Acta 542, 28–38.
[35] Bhuyan, K.C., Bhuyan, D.K. and Podos, S.M. (1991) Free Rad. Res. Commun. 12-13, 609–620.
[36] Bhuyan, D.K. and Bhuyan, K.C. (1991) Free Rad. Res. Commun. 12-13, 621–627.
[37] Spector, A. and Garner, W.H. (1981) Exp. Eye Res. 33, 673–681.
[38] Bhuyan, D.K., Camras, C.B., Lakhani, H.K. and Bhuyan, K.C. (1992) Invest. Ophthalmol. Vis. Sci. 33, 798 (abstr.).
[39] Bhuyan, D.K., Bergbauer, K.L., Yu, N.-T., Camras, C.B., Kuck, J.F.R., Jr. and Bhuyan, K.C. (1991) Invest. Ophthalmol. Vis. Sci. 32, 749 (abstr.).
[40] Esterbauer, H., Koller, E., Slee, R.G. and Koster, J.F. (1986) Biochem. J. 239, 405–409.
[41] Quehenberger, O., Koller, E., Jurgens, G. and Esterbauer, H. (1987) Free Rad. Res. Commun. 2, 233–242.