Susceptibility of rat retina acyl-CoA:1-acyl-sn-glycero-3-phosphocholine O-acyltransferase and CTP:phosphocholine cytidylyltransferase activity to lipid peroxidation and hydroperoxide treatment

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Abstract

Two enzyme activities involved in phospholipid metabolism in the rat retina were determined after in vivo and in vitro peroxidation according to several model systems. The in vivo models were based on: (i) intravenous administration of a sonicated emulsion of phospholipid and linoleate photooxidized mixture to normal rat for a period of one week; (ii) acute injection of Fe^{2+} solution (20 mM) or (iii) 0.5 mg of hydroperoxylinoleate into the vitreous body, and collection of retinal tissue 4 h or 4 days later, respectively. Oleoyl CoA:lysophosphatidylcholine acyltransferase activity was unchanged or exhibited significant inhibition. On the contrary, CTP:phosphocholine cytidylyltransferase activity was stimulated. By incubating in vitro the retina with: (i) Fe^{2+}-ascorbate; (ii) photooxidized phospholipid mixture (0.1–5 mM) or individual phospholipid classes; (iii) hydroperoxylinoleate (0.25–2 mM), with or without Fe^{2+}, a significant inactivation of acyltransferase (six-fold maximum loss of initial activity) and a slight stimulation of cytidylyltransferase were seen. Altogether, the results suggest that in situ oxygen radical generation by a variety of agents irreversibly perturbs enzymes and/or membrane structures in which the enzymes are inserted; these events may be a causal factor in retinal degeneration accompanying some ocular diseases.

Key words: Retina; Lipid peroxidation; Acyltransferase; Cytidylyltransferase; Phospholipids

1. Introduction

The retina, an organ with high oxygen demands, is particularly susceptible to light and antioxidant deficiency [1–3], and vulnerable to toxic oxygen radicals largely generated during its normal metabolism. The rat retina, and specifically photoreceptor membranes, are characterized by a high level (50–60 mol% of membrane fatty acids) of polyunsaturated fatty acids especially docosahexaenoic (more than 80%), esterified to phospholipid molecules [4]. The major protein of rod outer segments (ROS), rhodopsin, involved in the mechanism of the visual process, is buried in this highly fluid lipid bilayer forming the disc membranes. It is well known that polyenoic fatty acids undergo free radical peroxidation in many tissues, generating hydroperoxide groups in phospholipid acyl chains, and cytotoxic low molecular weight aldehydes reacting with amino groups of lipids and proteins. The hydroperoxides, released from phospholipids, deplete glutathione which further increases calcium influx and plasma membrane damage frequently leading to cell death. Loss of 22:6(n-3) is a common feature of the peroxidative process in the retina, concomitantly with an increase in conjugated dienes (CD) [1,2,5].

The expected susceptibility of retinal lipids to peroxidation may induce the inhibition of membrane functional capability, witnessed by alterations in resident enzyme activities. It is plausible that this phenomenon may also interest phospholipid-regulating enzymes. The ROS phospholipids are renewed by molecular replacement involving *in situ* exchange of fatty acids at positions 1 and 2 of the glycerol backbone. The presence of acylCoA:lysophospholipid acyltransferase activity (AT) in the retina has been demonstrated by several investigators [6,7]. This tailoring activity plays the role of locally replacing hydrolyzed, autooxidized acyl chains and giving ROS phospholipids their selective fatty acid composition. To the best of our knowledge, the indirect presence of phosphocholine cytidylyltransferase activity (CT), pertinent to de novo phosphatidylcholine (PtdCho) biosynthesis, has only once been suggested for retina preparations [8]. The reasons for this are not apparent, especially since this activity is rate-limiting of the metabolic pathway leading to PtdCho biosynthesis [9].

In an attempt to improve the understanding of the interaction between membrane-bound enzymes and the lipid bilayer, the present study focuses on the effect of lipid peroxidation on the two above-mentioned enzyme activities related to PtdCho biosynthesis in either in vitro or in vivo rat retinal preparations.

2. Materials and methods

2.1. In vivo models of peroxidation

Three models of retina tissue peroxidation were used.

(A) Firstly, we used a short-term model system in an attempt to

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generate prooxidant conditions in vivo in the rat retina. We thought that endogenous scavenging mechanisms might be overwhelmed by radical production occurring during repeated intravenous perfusions with an emulsion rich in peroxidized lipids. The model consisted in the repeated infusion of lipid emulsion through the femoral and/or jugular vein of adult Sprague–Dawley rats. Phospholipids from bovine brain were purified by column chromatography [10], and photooxidized by the method described by Terao et al. [11]. Crude phospholipids were dissolved in 5 ml of a mixture of CHCl₃/MeOH (1:1, v/v) containing 0.1 mM of Methylene blue followed by photoirradiation with a 100 W tungsten projector lamp, and bubbling O₂ in an open tube for 24 h at room temperature. After the peroxidation was complete, the reaction mixture was passed through a small column (1 × 8 cm) packed with Florisil (100–200 mesh) to remove dye, using CHCl₃/MeOH (1:1, v/v) as eluent. After evaporation in vacuum, the concentrated phospholipid mixture was suspended in saline, added with linoleate, peroxidized in the same way, and then sonicated under N₂ for 10 min using a Branson sonifier (Model 350) with 1 cm diameter probe tip at 90 W continuous power output in a constant temperature bath (4°C). Then, the emulsion produced was corrected to pH 7.35 with 1 M NaOH and again sonicated for 10 min. Hydroperoxides were determined according to Gebicki and Guille [12]. Two ml of a peroxidized mixture of phospholipids and linoleate (4:1, w/w; 500 mg/day; hydroperoxides: 200–250 nmol/mg lipid) thus prepared were intravenously administered to normal rats by insulin syringe for 20 min into the femoral or jugular vein of anesthetized animals, twice a day for a period of one week, at 9.00 h and 17.00 h. After the infusion, the rats had again free access to water and the same diet until their sacrifice. We did not find macroscopic lipidosis in any organ of any rats. At the end of one week treatment, retinas were excised, and tissue aliquots were used for enzyme assays and to measure the formation of malondialdehyde (MDA) and conjugated dienes (CD) [13]. Hydroperoxides were determined as above.

A group of five rats was i.v. injected with unoxidized phospholipid–linoleate mixture, using the same time and dose schedule described for peroxidized emulsion. In lipid-treated rats, none of peroxidation parameters we examined in the retina was changed compared to other groups of saline-injected animals. Therefore, in all experiments, groups of sham-operated, saline-injected animals were taken as controls.

(B) A second, acute in vivo model system consisted of a single injection (5 µl) of aqueous solution of Fe²⁺ ion (20 mM), into the vitreous body, 4 h before decapitation [5]. Control animals were intravitreally injected with 5 µl of saline.

(C) A third, acute in vivo model system consisted of a single intravitreal injection of hydroperoxylinoleate (0.5 mg/5 µl of artificial vitreous humor), 4 days before decapitation. Control animals were intravitreally injected with vehicle. In all the experimental model systems, retina tissue samples from 2–3 rats were pooled and homogenized with 10 mM Tris-HCl buffer, pH 7.4. Peroxidative damage to retina was monitored by measuring the tissue formation of MDA and CD, according to procedures described elsewhere [13].

2.2. In vitro peroxidation of retina tissue

Eyes from adult Sprague–Dawley rats, maintained in cyclic light, were removed rapidly and placed in ice-cold saline solution. The whole retina was carefully dissected free from the pigment epithelium under dim room light, homogenized with 10 mM Tris-HCl buffer, pH 7.4, and then treated with prooxidants in preincubation steps. First, an aqueous solution of Fe²⁺ (20 µM, final concentration)–ascorbate (0.25 mM, final concentration) was employed to generate hydroxyl radicals, at 37°C for 10 min. Second, unoxidized or peroxidized individual phospholipids or phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine plus phosphatidylinositol mixtures (55:35:10, molar%), dried under nitrogen, were dispersed by extensive sonication in 0.5 ml of the buffers used for enzyme assays. Immediately after sonication, the lipid emulsions were added (0.1–5 mM, final concentration) to unsonicated retina homogenates, and the resulting suspensions were vortexed and incubated at 37°C for 10 min. Third, retina homogenates were incubated with purified linoleate hydroperoxide alone (0.25–2 mM), or Fe²⁺ (100 µM) plus linoleate hydroperoxide (2 mM), at 37°C for 10 min (see [14]). Control incubations were performed without adding peroxidation inducers. Lipid peroxidation was assessed by MDA and CD measurements as above.

2.3. Enzyme assays

At the end of all the preincubation steps, the retina samples were immediately used for the enzyme determinations. Acyl-CoA:1-acyl-sn-glycero-3-phosphocholine O-acyltransferase (EC 2.3.1.23) was assayed using 25 µM [1-¹⁴C]oleoyl-CoA (Amersham, UK) and 50 µM lysophosphatidylcholine as substrates as described previously [13], except that the incubation buffer was 70 mM Tris-HCl buffer, 1 mM EDTA, 5 mM DTT, pH 7.4. After 10 min incubation, the reaction product (PtdCho) was estimated radiometrically following thin-layer chromatography separation of the lipid extract. CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) was assayed radiometrically according to Jamil and Vance [15], using phospho[Me-¹⁴C]choline (Amersham, UK) as substrate (1.5 mM). When the effect of phospholipids was tested, PtdCho/oleic acid (1:1 molar ratio) mixture was omitted from the assay mixtures. Additions to the incubations were carried out as specified in the legends of the figure and tables.

3. Results

Table 1 shows the effects of three in vivo peroxidative treatments on AT and CT activity. The slight increase in MDA values, suggesting a detectable presence of hydroperoxidized lipids in retina, paralleled AT inhibition and CT stimulation. The retina AT specific activity

Table 1
Effect of short-term i.v. treatment or single intravitreal injection of prooxidants on the phospholipid synthesis-related enzyme activities of rat retina

Treatment	MDA (nmol/100 mg protein)	Acyltransferase (nmol/min/mg protein)	Cytidylyltransferase (nmol/min/mg protein)
Vehicle (saline)[a]	7.9 ± 2.4	1.00 ± 0.12	1.84 ± 0.32
Peroxidized emulsion, i.v. (1 week)[b]	8.1 ± 1.9	0.71 ± 0.11*	2.85 ± 0.64*
Vehicle (artificial vitreous humor)	8.8 ± 2.1	0.92 ± 0.24	2.00 ± 0.52
Fe²⁺ (4 h)	19.2 ± 5.8*	0.60 ± 0.08*	1.17 ± 0.30*
Linoleate (4 days)	9.6 ± 2.7	0.96 ± 0.25	2.81 ± 0.62*
Linoleate hydroperoxide (4 days)	11.6 ± 3.4	1.21 ± 0.31	2.56 ± 0.47

Four hours after the last intravenous injection of the sonicated emulsion of peroxidized phospholipid–linoleate mixture (lasted for one week, twice a day), 4 h after intravitreal injection of Fe²⁺ or 4 days after a single intravitreal injection of pure linoleate or linoleate hydroperoxide, the retinas were excised and aliquots used for the determination of MDA (TCA supernatant) and CD content as well as for enzyme activities as described in the text. Results are the average of three separate experiments with S.D. indicated.
By ANOVA, *P < 0.05 for significant difference from control retina of untreated rats.
[a] CD mean value was 7.8 ± 0.7 nmol/mg protein.
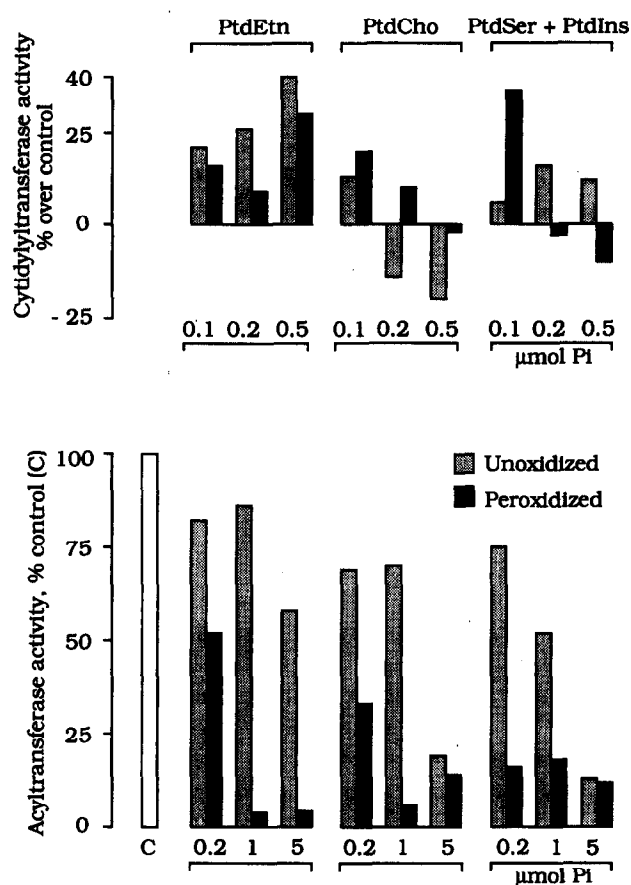[b] CD mean value was 8.6 ± 0.8 nmol/mg protein.

Fig. 1. Dose-dependent effect on cytidylytransferase and acyltransferase activities of the rat retina induced by membrane replacement with phospholipid classes. PtdEtn, PtdCho and PtdSer + PtdIns were purified from bovine brain total lipid extract and peroxidized as described in Experimental Procedures. Fusion of retina homogenates with exogenous phospholipids was performed by incubating the tissue preparations (100 μg protein) for 10 min at 37° with emulsions in 10 mM Tris-HCl, 1 mM EDTA buffer (pH 7.4), containing increasing amounts (μmol inorganoc P_i) of each unoxidized or peroxidized phospholipids, sonicated twice for 10 s at room temperature. Control assays mixtures (C) were preincubated in the absence of phospholipids. Pretreated retina prepa-rations were readly assayed for CT and AT reactions by diluting them into the incubation medium, and following then CT activity for 30 min, and AT activity for 10 min. Two μmol of either unoxidized or peroxidized phospholipid of any kind, added to 100 vg retina protein, caused strong inhibition of CT activity, whereas 10 μmol drastically inhibited AT activity. Each data point is the mean of two independent experiments in which the variation was less than 15%.

reached significant inhibition after one week intravenous treatment with peroxidized lipid emulsion and intravitreal iron-injection, whereas it did not change significantly in hydroperoxylinoleate-injected eye. In contrast, retina CT specific activity significantly increased after one week intravenous treatment. Intravitreal injection of linoleate or linoleic acid hydroperoxide also promoted stimulation of the enzyme activity. However, it showed a marked decrease in Fe^{2+} acute model.

Table 2 shows the effect in vitro of various free radical

generating systems on AT and CT activity. In the retinal homogenates, MDA formation markedly increased after incubation with hydroxyl (Fe^{2+}/ascorbate) or peroxyl (hydroperoxides) radical inducers, even during incubation with pure linoleate or unoxidized phospholipids owing to the presumable presence of metal ion impurities. AT activity was always inhibited (sixfold maximum loss for linoleate hydroperoxide at 2 mM concentration) whereas CT activity was unchanged or stimulated (56% increase for Fe^{2+}/ascorbate treatment). The presence of Fe^{2+} in the incubation system caused an increase in retina lipid peroxidation induced by either ascorbate or linoleate hydroperoxide (see [14]). Previous research demonstrated that cumene hydroperoxide (1–10 mM) inhibits ROS acyltransferase and phospholipase A activity in vitro [16].

Fig. 1 illustrates the slight CT stimulation, compared to untreated control, induced either by 0.1–0.5 μmol of unoxidized or peroxidized phosphatidylethanolamine (PtdEtn) added to 100 μg retinal protein. Both forms of purifed PtdCho and phosphatidylserine (PtdSer) plus phosphatidylinositol (PtdIns) caused activation at 0.1 μmol dose, but slight inhibition at higher doses (0.2–0.5 μmol). AT showed a marked inactivation with 0.2–5 μmol of peroxidized PtdEtn, PtdCho or PtdSer plus PtdIns. Although the effect of peroxidized form was much more drastic, the susceptibility of the enzyme activity to additions of unoxidized and peroxidized phospholipid classes increased with their increasing amount until they reached the same level, i.e. 5 μmol of PtdCho or acidic phospholipids. The inhibitory effect by peroxidized PtdCho, at equivalent amount, was lower than PtdEtn, whereas the addition of PtdSer+PtdIns was almost as effective as PtdCho (see [13]).

4. Discussion

This study highlights some aspects of lipid peroxidation effect on membrane-bound enzymes associated with phospholipid synthesis. The main observation is that acyltransferase activity is inhibited in every in vivo and in vitro peroxidation model used, whereas cytidylyltransferase activity was slightly stimulated or unaffected by membrane-incorporated hydroperoxides. We suggest that this apparently conflicting result could be a reflection of the possible difference in the structure and membrane location of the two enzymatic proteins. AT could be deeply embedded in the hydrophobic core of microsomal membranes while CT could be loosely associated with the cytoplasmic side [17]. Membrane bilayer is extensively disrupted during incubation with prooxidant or the action of exogenously injected hydroperoxides such that AT might undergo vertical translocation, its thiol-groups exposed and oxidized. The chemical nature of phospholipid hydrophobic moieties (hydroperoxy-, hy-

droxy or unoxidized acyl chains) in the vicinity of the membrane enzyme instead seems not an important structural determinant for expressing CT activity. When the membrane becomes rigid due to pe-roxidation events [18], the CT molecule might keep sulfhydryl groups protected and become more accessible to hydrophilic substrate.

In the model much used in the present study, lipid hydroperoxides, intravenously injected, are transported in the systemic circulation by lipoproteins. When taken up by the endothelial cells of pigment epithelium choriocapillaries and the microvasculature of the inner portion of the retina, they produce structural damage to the blood-retina barrier, and particularly to the photoreceptor layer where peroxidase is absent [19]. The membrane structural integrity could be strikingly damaged by substantial peroxidation as seen by the loss of disk membrane morphology under the electron microscope [20,21]. Moreover, AT inhibition essentially brings about an accumulation of highly cytotoxic lysophospholipids, the products of activated phospholipases [16,22]. It should be pointed out that the retina possesses antioxidants and protective enzymes against autooxidation. The levels of α-tocopherol [20], ascorbic acid [24], reduced gluta-thione [25], and the specific activity of superoxide dismutase are very high [24,26]. However, the low activities of catalase [19,27] and gluta-thione peroxidase [19,28–30] compared to those of brain and other tissues, might hardly balance an intense oxidative stress through scavenging H_2O_2 and lipid hydroperoxides. This meagre enzymatic frame favors the accumulation of peroxides and consequent cell damage in the retina.

CT was differently affected by Fe^{2+} ions in the in vivo (marked increase) and in vitro (marked decrease) experiments. Two considerations might help to explain the conflicting results. In frogs and rats, intravitreal treatment with Fe^{2+} massively disrupts membrane integrity within 4 h, and causes more drastic and faster structural and functional alterations in the photoreceptor cells, retina inner layer and pigment epithelium [5,31] than treatment with linoleate, hydroperoxylinoleate [23] or peroxidized emulsion. This is also consistent with the highest MDA levels we found in the Fe^{2+}-model, in agreement with in vitro retina incubations with prooxidants for a short duration. In addition, the free radical mechanisms by which intravitreal Fe^{2+} and hydroperoxylinoleate overloading might act are quite different. The former generates, by Fenton's reaction and presumably intraretinal ascorbate, hydroxyl radical which has been implicated [32] in in situ extensive damage of enzymes and/or membranes as function of its continuous production. The latter, incorporated into membrane phospholipids, is largely reduced to unreactive hydroxylinoleate by glutathione peroxidase. This mechanism limits the damage by bilayer-penetrating lipoperoxy radical (LOO•), which is generated only in the in vivo presence of transition metals or iron complexes. Moreover, at low fatty acid peroxide concentrations, phospholipase A_2 protects membranes from their damaging effects by removing them from phospholipids [22,33].

The metabolic changes observed in the present study appear to be pathogenetic for retinal diseases and retinal function during disease processes such as diabetes, inflammation, exposure to oxidants, metal ions or light,

Table 2
Effect of Fe^{2+} and hydroperoxides on phospholipid synthesis-related enzyme activities of rat retina in vitro

Treatment	MDA (nmol/100 mg protein)	Acyltransferase (nmol/min/mg protein)	Cytidylyltransferase (nmol/min/mg protein)
None[a]	10.3 ± 3.9	0.67 ± 0.15	1.04 ± 0.21
Fe^{2+} (20 μM)/ascorbate (250 μM)[b]	137.5 ± 22.7**	0.33 ± 0.11*	1.61 ± 0.32*
Linoleate (2 mM)	33.3 ± 6.1*	0.11 ± 0.02**	1.47 ± 0.43
Linoleate hydroperoxide (250 μM)	28.9 ± 7.8	0.22 ± 0.03**	1.35 ± 0.20
Linoleate hydroperoxide (2 mM)	34.6 ± 8.2*	0.10 ± 0.01**	1.25 ± 0.31
Fe^{2+} (100 μM)/linoleate hydroperoxide (2 mM)	54.1 ± 11.9**	0.11 ± 0.03**	1.17 ± 0.17
Phospholipids (0.1 mM)	10.7 ± 2.1	0.61 ± 0.09	1.66 ± 0.30*
Phospholipids (1 mM)	11.9 ± 2.8	0.54 ± 0.08	1.65 ± 0.34*
Photooxidized phosphlipids (0.1 mM)	13.5 ± 3.1	0.26 ± 0.04**	1.67 ± 0.40*
Photooxidized phospholipids (1 mM)	21.4 ± 6.4	0.24 ± 0.05**	1.70 ± 0.42*
Photooxidized phospholipids (5 mM)	30.7 ± 6.7	0.16 ± 0.02*	0.92 ± 0.25

Incubations of excised retina (2 mg protein/2 ml) were conducted in a medium containing 10 mM Tris-HCl, 1 mM EDTA (pH 7.4), following 10 min exposure at 37°C to the indicated conditions. The remaining reagents or extraction solvents were added to aliquots of the incubation mixture to determine CD and enzyme activities by the radiometric methods described in the text. MDA production was determined in the supernatant obtained after protein precipitation with 12% TCA. Unoxidized or photooxidized phospholipids were a mixture of phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine + phosphatidylinositol in the molar ratio 55:35:10. All figures represent the mean ± S.D. of the enzyme persisting after 10-min incubation, with $n = 3$.
By ANOVA, *$P < 0.05$ or **$P < 0.001$ for significant difference from untreated control retinas.
[a] CD mean value was 6.4 ± 1.2 nmol/mg protein. For retina homogenate without preincubation, 10 min at 37°C, acyltransferase and cytidylyltransferase specific activities were 0.98 and 1.93 nmol/min/mg protein, respectively.
[b] CD mean value was 14.5 ± 3.7** nmol/mg protein.

and ischemia-reperfusion injury, or to aging [1,2,5,34,35] which share lipid peroxidation by reactive oxygen species as a common feature.

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