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Lipid peroxidation of membrane phospholipids in the vertebrate retina

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1. ABSTRACT

Retina is very rich in membranes containing polyunsaturated fatty acids. Reactive oxygen species initiates chain reactions of lipid peroxidation which injure the retina, especially the membranes that play important roles in visual function. Furthermore, biomolecules such as proteins or amino lipids can be covalently modified by lipid decomposition products. In retinal membranes, peroxidation of lipids is also usually accompanied by oxidation of membrane proteins. In consequence, lipid peroxidation may alter the arrangement of proteins in bilayers and by that interfere with their physiological role on the membrane function. Here, we review several studies on the lipid peroxidation of membrane phospholipids in retina. Particular emphasis is placed on the molecular changes of very long chain polyunsaturated fatty acids associated with protein modifications during peroxidation of photoreceptor membranes**.** Furthermore we use liposomes to analyze peroxidation of retinal lipids. Conjugated dienes formed from oxidized PUFAs, and TBARS products derived from the breakdown of these fatty acids located in phospholipids can be analyzed during lipid peroxidation of liposomes made of retinal lipids using Fe^{2+} and Fe^{3+} as initiators.

2. INTRODUCTION

Peroxidation of polyunsaturated fatty acids (PUFAs) in lipid bilayer membranes causes loss of fluidity, a fall in membrane potential, increased permeability to protons and calcium ions, and eventually, breakdown of cell membranes because of cellular deformities. The structural and functional integrity of the cell membranes is necessary for signal transduction, molecular recognition and transport, cellular metabolism, etc. The damage inflicted upon biological systems by reactive oxygen species have been implicated in numerous disease processes including inflammation, degenerative diseases tumor formation and involved in physiological phenomena such as aging. Initiation is the most important phase of lipid peroxidation especially in a cellular context; preventive therapy of lipid peroxidation-associated disease would target the initiation process. Indeed, many ocular disorders including glaucoma, cataracts, diabetic retinopathy and retinal degeneration have been attributed to lipid peroxidation processes. Because of intense exposure to light and oxygen and their high PUFA content which is prone to lipid peroxidation, the retina is highly susceptible to oxidative stress.

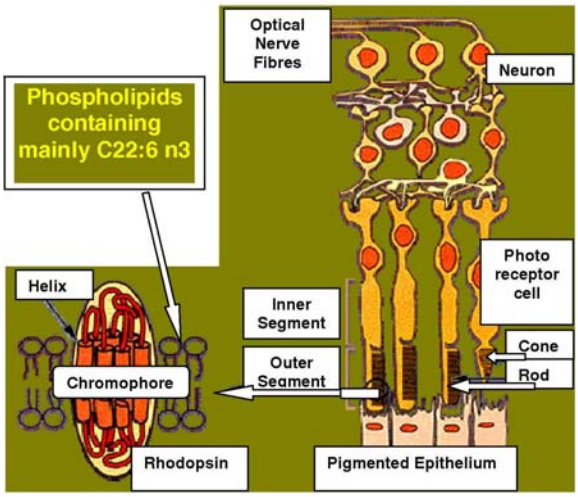

Figure 1. Schematic diagram of rhodopsin in the membrane of the photoreceptor cell.

3. PHOSPHOLIPID SPECIES IN THE RETINA

Lipids represent approximately 20% of dry weight of bovine retina, of which about two-thirds are phospholipids. Although existing data are sparse, nonpolar lipids (i.e. acyl glycerides, sterols, and free fatty acids) account for about one-fourth of total retinal lipids. In general, ratio (wt/wt) of nonpolar lipid to phospholipids is in the range 0.3-0.5. Phosphatidylcholine (40-50 %) and phosphatidylethanolamine (30-35 %) account for the majority of phospholipids, with lesser amounts of phosphatidylserine (5-10 %), phosphatidylinositol (3-6 %) and sphingomyelin (2-8 %). Phosphatidic acid and cardiolipin are very minor phospholipid constituents (1).

3.1. Very long chain polyunsaturated fatty acids in the retina

The major fatty acids (wt %) of bovine retinas are 16:0 (≈25 %), 18:0 (≈17 %), 18:1 (≈17 %), and a 22-carbon polyunsaturated acid (≈23 %) which was later identified as all-cis docosahexaenoic acid (22:6 n3) by Hands and Bartley (2). Over 50 % of total bovine retina fatty acids are unsaturated, of which polyunsaturated acids account for at least 60 %. Lipids containing the very long chain polyunsaturated fatty acid docosahexaenoic (DHA 22:6n3) are found at high concentrations in brain synaptosomes and the retina. They are essential for the development of the human brain (3).

The retina contains very high levels of 22:6n-3 representing the highest concentration of PUFAs of any vertebrate tissue (4). In fact, 50% of all acyl chains in the outer segments of photoreceptors phospholipids (both sn-1 and sn-2) are 22:6n-3 (in PC, PE, and PS). Minor phospholipids, like phosphatidylinositol and phosphatidic acid, contain predominantly 20:4n-6 (5). Thus, rod outer segments represent an excellent model to define the role of 22:6n-3 in membrane structure and function. It have been suggested that polyunsaturation alters membrane properties that are critical for activity of integral receptor proteins (6). Therefore, exploring the structure of polyunsaturated bilayers is a prerequisite for understanding how neural membranes function. It has been demonstrated that distribution of saturated and polyunsaturated hydrocarbon chains differs significantly, supporting the hypothesis that DHA-containing membranes are under considerable elastic stress that may influence the function of integral membrane proteins (7).

The rod outer segment (ROSg) membranes are essentially lipoprotein complexes. Rhodopsin, the major integral protein of ROSg, is surrounded by phospholipids highly enriched in docosahexaenoic acid (C22:6 n3), Figure 1.

This fluid environment plays an important role for conformational changes after photoactivation. Thus, ROSg membranes are highly susceptible to oxidative damage. The most careful studies on the effects of DHA-enriched diets have been performed on the visual system because DHA is a major constituent of photoreceptor membranes (8, 9). In retina, a slight reduction in DHA content in membrane phospholipids has a critical effect on the renewal of new photoreceptor discs (10). To produce gross DHA deficiency, it is necessary to deprive rats of n-3 fatty acids during development and throughout life for more than two generations. Supplementation of rats with an n-3 fatty acid enriched diet results in normalization of retinal and occipital cortex DHA contents. These changes are reflected in alterations in the electroretinogram and visual acuity tests in human and nonhuman primates (11). Thus DHA induced changes in neural membrane fatty acid composition may lead to restoration of many membrane properties such as membrane fluidity, receptor affinities, ion fluxes, and activities of membrane-bound enzymes.

3.2. Rod outer segments of retina are susceptible to lipid peroxidation because of their high content of docosahexaenoic acid

Oxidative stress has been proposed as a possible cause of the progression of AMD. (12–19). The retina is particularly susceptible to oxidative stress because of its high metabolic activity, oxygen tension, and concentration of easily oxidized polyunsaturated fatty acids (PUFAs), as well as the presence of retinal pigments that generate reactive oxygen species when illuminated by light (20).

Rod outer segments (ROSg) of retina are susceptible to lipid peroxidation because of their high content of PUFAs, mainly docosahexaenoic acid (C22:6 n3) (21). It has been suggested that lipid peroxidation participates in the oxidative damage leading to retinal degeneration. The lipid peroxidation process proceeds via radical chain reaction resulting in the formation of lipid hydroperoxides (LOOH). Lipid peroxidation is a complex system where the generation of the initiator molecule is followed by chain initiation, propagation, branching and termination reactions, Figure 2. Numerous studies have implicated the hydroxyl radical in the initiation of lipid peroxidation However; there are reports that, in some experimental systems, the hydroxyl radical is not involved in the initiation step. The short-lived nature of the hydroxyl

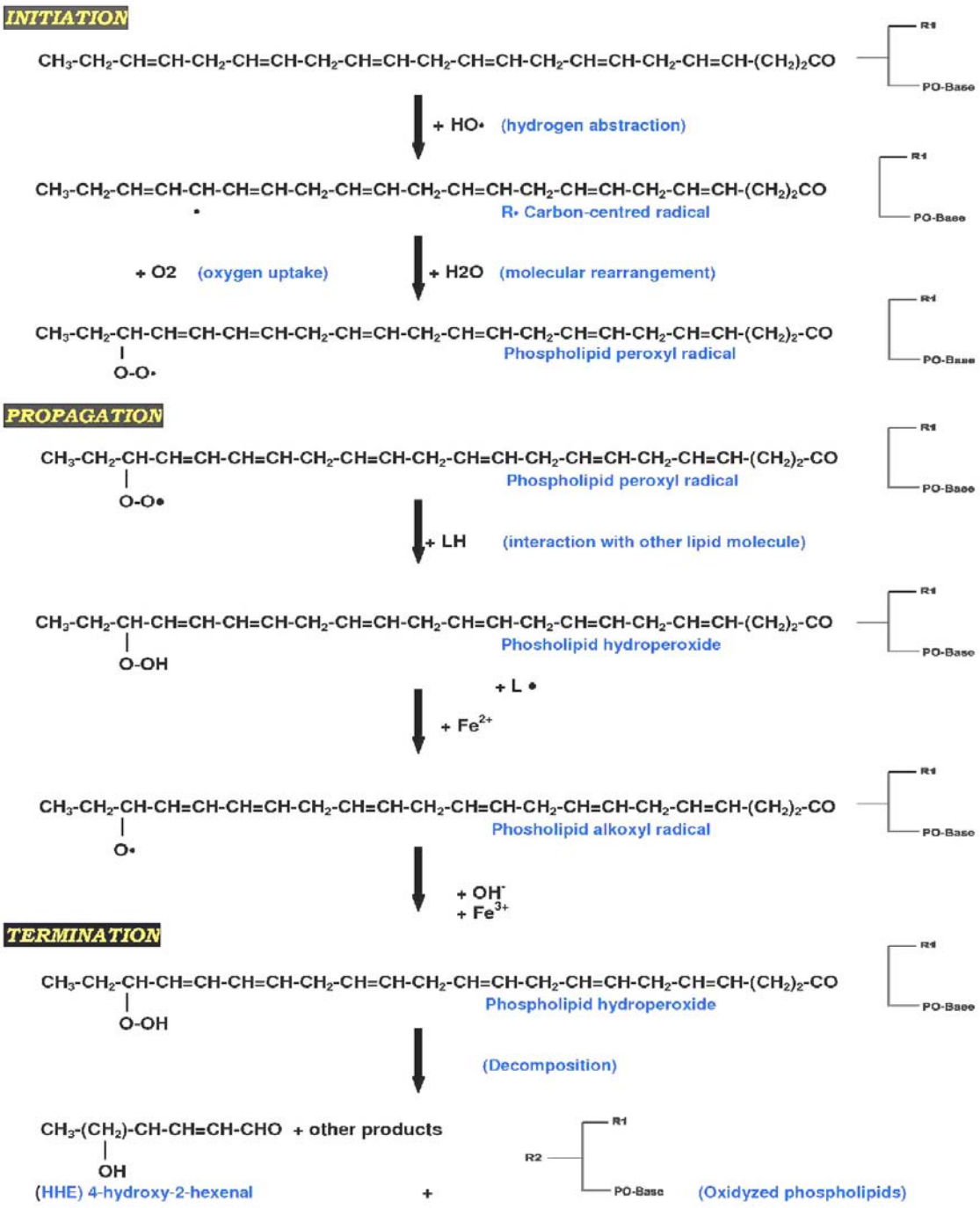

Figure 2. Chemical diagram of the steps in lipid peroxidation of phospholipids containing docosahexaenoic acid (22:6 n-3), R1 = fatty acid, R2 = fragmentation products of fatty acid oxidation.

radical makes it unlikely that it could migrate from the site of generation to the hydrophobic membrane interior where peroxidation must be initiated. Lipid hydroperoxides derived from unsaturated phospholipids, glycolipids and cholesterol are prominent nonradical intermediaries of lipid peroxidation and perturb membrane structure and function with cytopathological consequences. Because of their increased polarity and long lifetime compared with free radical precursors, long-chain fatty acid hydroperoxides may be able to migrate from points of origin to more sensitive sites. Such movements might be spontaneous or facilitated by lipid transfer proteins (22). The retina can generate lipid hydroperoxides through enzymatic oxidation of endogenous PUFAs, which are intermediaries in the reactions to form docosanoids (23). As demonstrated by De La Paz and Anderson, the hydroxyl radical is unlike to be the initiator of the LPO in ROSg membrane (24). These

authors postulate that endogenous hydroperoxides form more stable free-radical species in comparison with the hydroxyl radical. There is resultant facilitated entry of these species into the interior of the bilayer to initiate peroxidation of long fatty acyl chains.

We have previously found that the peroxidation of ROS by the ascorbate/iron system is greatly enhanced in the presence of PUFA hydroperoxides and that the ability of these lipids to stimulate chemiluminescence strongly depends on the degree of unsaturation and concentration. Our results suggest that lipid hydroperoxides in the retina can serve as a source of lipid free radicals and promote peroxidation of the long-chain PUFAs (25)

3.3. Peroxidation of lipids in retina is accompanied by oxidation of membrane proteins

Lipids containing polyunsaturated fatty acids are susceptible to free radical–initiated oxidation and can contribute in chain reactions that amplify damage to biomolecules as described above. Lipid peroxidation often occurs in response to oxidative stress, and a great diversity of aldehydes is formed when lipid hydroperoxides break down in biological systems. Some of these aldehydes are highly reactive and may be considered as second toxic messengers, which disseminate and augment initial free radical events. The aldehydes most intensively studied up to now are 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal, and malondialdehyde. 4-hydroxy-2-nonenal (HNE) is known to be the main aldehyde formed during lipid peroxidation of n-6 polyunsaturated fatty acids, such as linoleic acid C18:2 n-6 and arachidonic acid C20: 4 n-6, (26).

On the other hand, lipid peroxidation of n-3 polyunsaturated fatty acids such as α-linolenic acid C18:3 n-3 and docosahexaenoic acid C22:6 n-3 generates a closely related compound, 4-hydroxy-2-hexenal (HHE), which is a potential mediator of mitochondrial permeability transition (27). 4-hydroxy-2-alkenals represent the most prominent aldehyde substances generated during lipid peroxidation. Among them, 4-hydroxy-2-nonenal (HNE) is known to be the main aldehyde formed during lipid peroxidation of n-6 polyunsaturated fatty acids, such as linoleic acid and arachidonic acid, Figure 3.

4-hydroxynonenal (HNE) was identified three decades ago as a cytotoxic aldehyde formed during the NADPH-Fe^{++} induced peroxidation of liver microsomal lipids (28). Since then, a vast number of reports have been available, which sustain a function for this compound in a diversity of disease processes. HNE is considered as an indicator of oxidative stress and a probable contributing agent of several diseases.

Guajardo *et al* (21) have studied the changes in the ROSg membranes isolated from bovine retina submitted to nonenzymatic lipid peroxidation, during different periods of time. Oxidative stress was monitored by increase in the chemiluminescence and fatty acid alterations. In addition they studied the *in vitro* protective effect of 5 mM melatonin. The total cpm originated from light emission (chemiluminescence) was found to be lower in those membranes incubated in the presence of melatonin, Figure 4.

The docosahexaenoic acid content decreased considerably when the membranes were exposed to oxidative damage and this was viewed by changes in the unsaturation index UI at different intervals of lipid peroxidation. The reduction of C22:6n3 was from 35.5 +/-2.9% in the native membranes to 12.65 +/-1.86% in those peroxidized during 180 min. In the presence of 5 mm melatonin it was observed a content preservation of 22:6 n3 (23.85 +/-2.77%) at the same time of peroxidation. Simultaneously the alterations of membrane proteins under oxidative stress were studied using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Loss of protein sulfhydryl groups and increased incorporation of carbonyl groups were utilized as biomarkers of protein oxidation. In membranes exposed to Fe^{2+} -ascorbate, they observed a decrease of protein thiols from 50.9 +/-3.38 in native membranes to 1.72 +/-2.81 nmol/mg of protein after 180 min of lipid peroxidation associated with increased incorporation of carbonyl groups into proteins from 7.20 +/-2.50 to 12.50 +/- 1.12 nmol/mg of protein. In the SDS-PAGE a decrease in the content of all the proteins was observed, mainly rhodopsin, as a consequence of peroxidation Figure 5.

4. THE RETINA AS A MODEL TO STUDY LIPID PEROXIDATION

4.1. Liposomes as a tool to analyze peroxidation of retinal lipids

Biological membranes in retina are complex systems. In view of this complexity and in order to evade collateral consequences that may take place during lipid peroxidation of entire retinal membranes, we have tried to increase understanding of the mechanisms responsible for oxidation in simple model systems, made by dispersing retinal lipids in the form of liposomes. In such systems it is possible to check peroxidation under different conditions while varying the factors that govern the reaction in a convenient manner, one at a time.

Relatively simple liposomal model membranes are still rather intricate, but unlike biological membranes, they facilitate evaluation of the effects of different prooxidantes, varying lipid composition and/or arrangement of membranes on consequences of lipid peroxidation. Liposomes, in which phospholipid composition, structure and dynamics can be completely controlled, are frequently accepted to be an appropriate model for *in vitro* studies of membrane structures and properties. They are surrounded by a lipid bilayer, structurally similar to cell membrane lipidic environment (29, 30).

Phospholipid vesicles are often used as model systems to study the physical principles behind the activities of biological membranes. Conjugated dienes are formed from the double-bond reorganization of oxidized PUFAs, and TBARS are products resulting from the breakdown of these fatty acids located in phospholipids.

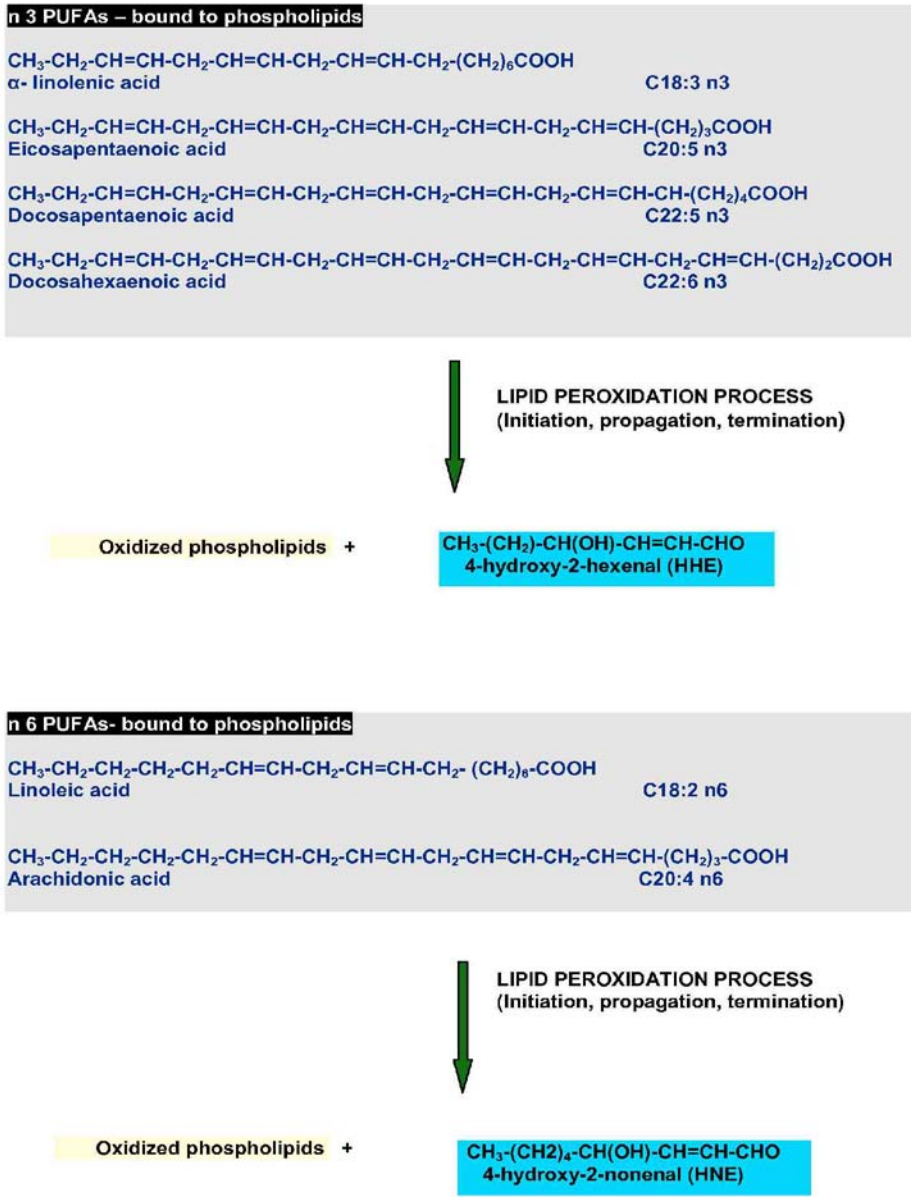

Figure 3. Schematic diagram of reactive hydroxy-alkenals generated during lipid peroxidation of n-3 and n-6 polyunsaturated fatty acids.

We have investigated the performance of these compounds during evolution of lipid peroxidation of sonicated and non-sonicated liposomes made of retinal lipids in different aqueous media using Fe^{2+} and Fe^{3+} as initiators (31). This model system resembles the characteristics of a biological membrane better than simple chloroform lipid solutions as we employed in the past (32, 33).

As initiator of lipid peroxidation, we used Fe^{2+} or Fe^{3+}, which produced free radical species in the presence of LOOHs. LOOH-dependent initiation has been proposed to occur by two pathways: LOOH breakdown by Fe^{3+} and subsequent hydrogen abstraction by LOO• (reactions 1 and 2)

$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO\bullet + H^{+}$ (1)
$LOO\bullet + LH \rightarrow L\bullet + LOOH$ (2)

or LOOH breakdown by Fe^{2+} to free radicals (reactions 3 and 4)

$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\bullet + OH^{-}$ (3)
$LO\bullet + LH \rightarrow L\bullet + LOH$ (4)

In compartmentalized systems such as liposomes, it is useful to assume that free radical inducers, such as Fe^{2+} and

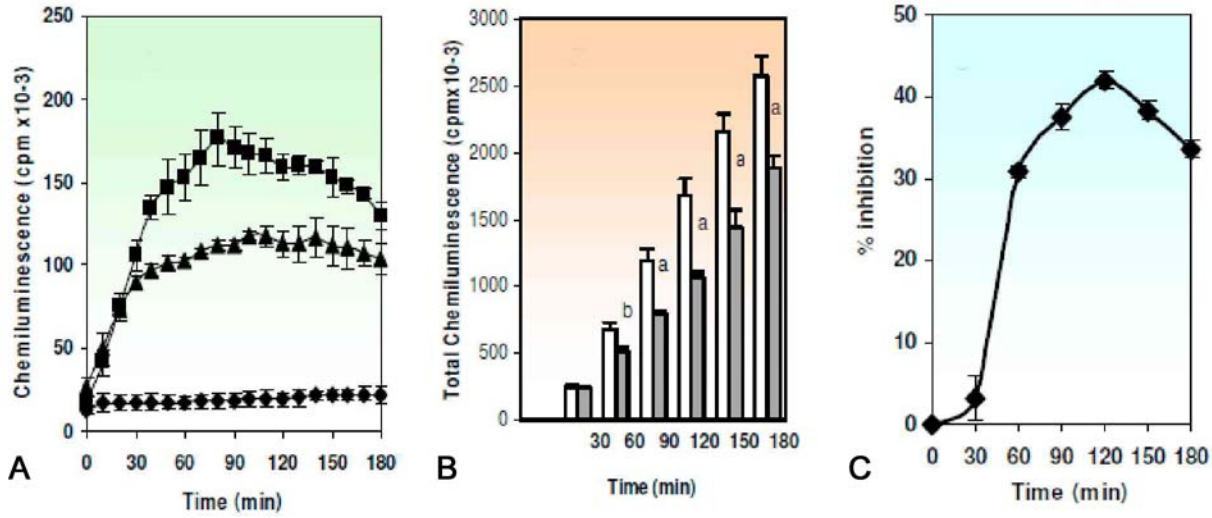

Figure 4. A- Chemiluminescence as a function of time during Fe^{2+}-ascorbate lipid peroxidation of ROSg membranes (0.5 mg of protein). Effect of melatonin. (-•-) control without ascorbate; (-■-) peroxidized with 0.4mM ascorbate and (-▾-) peroxidized with 0.4 mM ascorbate plus 5 mM melatonin. B- Total chemiluminescence produced by ROSg membranes (0.5 mg of protein) recorded as cpm every 10 min during different intervals of time of peroxidation (30, 60, 90, 120, 150 and 180 min). Effect of melatonin (open bars) with 0.4 mM ascorbate, (closed bars) with 0.4 mM ascorbate plus 5 mM melatonin. Data are mean ± S.E.M. of three independent experiments. Statistically significant differences between peroxidized versus peroxidized + melatonin groups are indicated by ^{a}P <0.005 and ^{b}P<0.05. C- Percent inhibition of total chemiluminescence by 5 mM melatonin as a function of lipid peroxidation-time. The zero percent of inhibition was calculated by subtracting the total cpm originated by control without ascorbate to peroxidized ROSg membranes (with ascorbate). The percent of inhibition for each time was calculated by comparing the total cpm with the zero percent. Reproduced with permission from Guajardo *et al*, 2006.

Fe^{3+} used in our study, located in the external medium should first achieve admittance to the unsaturated fatty acyl chains buried in the interior of the membrane bilayer to begin the chain reaction of lipid peroxidation. If this is the case, both the transition metal ions and oxygen must penetrate into the membrane bilayer and higher water permeability of the latter would surely aid this process. Numerous physical studies on the acyl chain structure of phospholipid bilayer vesicles propose that acyl chain packing depends in part on the radius of curvature of the vesicles (34, 35).

5. LIPID PEROXIDATION PARTICIPATES IN THE OXIDATIVE DAMAGE LEADING TO RETINAL DEGENERATION

Retina is particularly susceptible to oxidative stress because not only it is attacked constantly by ROS-producing UV and high-energy visible light (36), but also because retinal pigment epithelial (RPE) cells preserve and maintain the photoreceptors by phagocytosis and degradation of the photoreceptor outer segment membranes which are rich in polyunsaturated fatty acids (37-39). It has been suggested that LPO products contribute to retinal pigment epithelial dysfunction, initiating retinal degenerative disorders including age-related macular degeneration (ARMD) which is the principal cause of blindness in the developed world (40). 4-Hydroxy-2-trans-nonenal (4-HNE), one of the major end products of lipid peroxidation, has been shown to induce apoptosis in a variety of cell lines. It appears to modulate signaling processes in more than one way because it has been suggested to have a role in signaling for differentiation and proliferation. Shrama *et al* studied the effects of 4-HNE on the expression and activation of p53 in RPE cells focusing on the p53-mediated intrinsic pathway for apoptosis (40). Glutathione *S*-transferase A4-4 (GSTA4-4)-mediated metabolism of 4-HNE is one of the major determinants of the intracellular concentration of 4-HNE [41-43]. Therefore, the authors examined the possible role of GSTA4-4 in regulation of 4-HNE-induced, p53-mediated apoptosis in RPE cells. For these studies, they have chosen RPE cells of human fetal origin and ARPE-19 cells developed from the retina of adult young male. Results of these studies indicate that in these cells 4-HNE causes activation, phosphorylation, and enhanced nuclear accumulation of p53, accompanied with activation of the signaling components involved in p53-mediated apoptosis. Over-expression of human GSTA4-4 or the corresponding murine isozyme mGsta4-4 as well as the silencing of cellular p53 blocks these effects of 4-HNE, these studies suggest that alterations in 4-HNE homeostasis can profoundly affect cell-cycle signaling events.

6. PERSPECTIVE

The addition of oxygen to lipids is an important process developed by biological systems to produce a broad spectrum of compounds both by enzymatic and non-enzymatic mechanisms. The abundant content of polyunsaturated fatty acids in the retina makes this tissue particularly susceptible to peroxidation. Lipid peroxidation

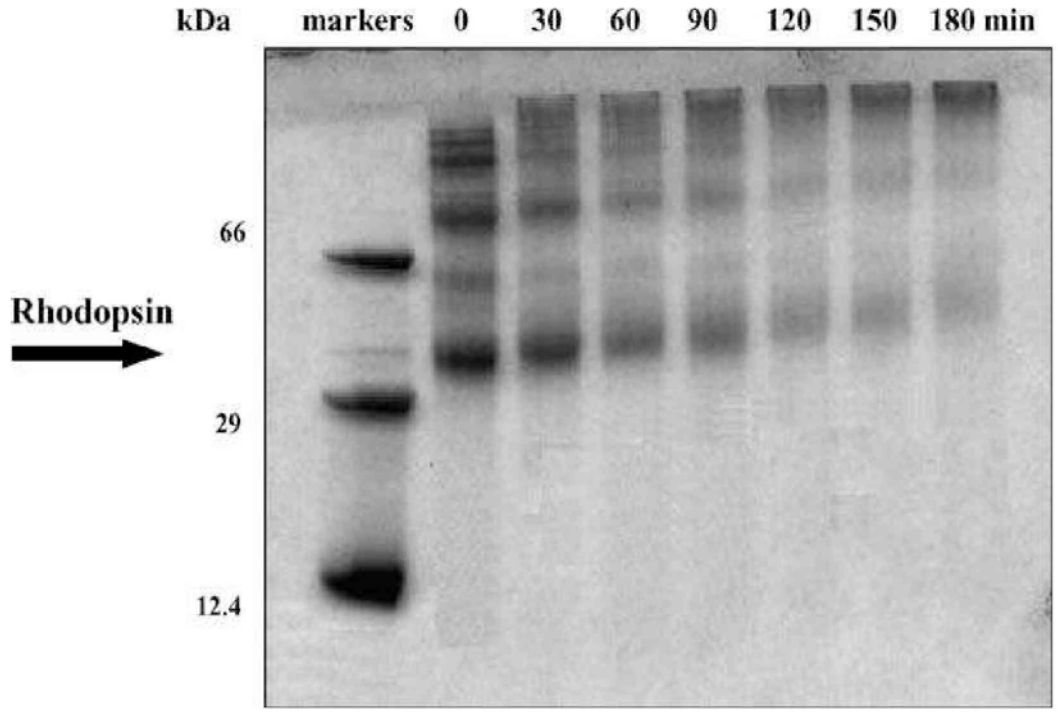

Figure 5. SDS-PAGE of samples containing rod outer segment membranes peroxidized during different intervals of time.

exerts a significant role in the origin of several retinopathies. Taken together; the evidence suggests that oxidative stress is involved in the pathogenesis of retinopathies possibly by oxidizing phospholipids in the photoreceptors as demonstrated in the arterial intima of patients with atherosclerosis. It is likely that controlling oxidation of phospholipids may be a potential treatment for eye diseases. Although strong evidence has accumulated that oxidative stress plays a key role in the pathogenesis of several retinopathies, it has not been directly demonstrated how the oxidative stress contributes to the development and progression of these diseases. Further studies are needed to determine the exact pharmacological role of lipid derived free radicals in eye diseases.

7. ACKNOWLEDGMENTS

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Abbreviations: ARMD, age-related macular degeneration; CPM, counts per minute; PUFAs, polyunsaturated fatty acids; 18:2n-6, linoleic acid; 18:3n-3, α-linolenic acid: 20:4n-6, arachidonic acid; 22:6n-3 (DHA), docosahexaenoic acid; HHE, 4-hydroxy-2-hexenal; HNE, 4-hydroxy-2-nonenal; LCPUFA, long chain polyunsaturated fatty acids; LOOH, lipid hydroperoxides;, PL, phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ROSg, rod outer segments; TBARS, thiobarbituric acid reactive substances.

Key Words: Retina; Lipid peroxidation; Review

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