Vitamins C and E Reduce Retinal Oxidative Stress and Nitric Oxide Metabolites and Prevent Ultrastructural Alterations in Porcine Hypercholesterolemia

Patricia Fernandez-Robredo,¹ David Moya,¹ Jose Antonio Rodriguez,² and Alfredo Garcia-Layana¹

PURPOSE. Oxidative stress is thought to be involved in the pathogenesis of age-related diseases, such as atherosclerosis and retinal degeneration. The current study was conducted to examine vitreoretinal oxidative status in a model of porcine hypercholesterolemia to identify morphologic alterations and analyze the effect of dietary supplementation with vitamins C and E.

METHODS. Adult miniature pigs were fed standard chow, cholesterol-rich chow, or a cholesterol-rich diet supplemented with vitamins C and E. Total cholesterol, triglycerides, high-density lipoproteins, lipid peroxidation, and tocopherol were measured in plasma. Lipid peroxidation and nitric oxide (NO) metabolites were measured in vitreous and retinal homogenates. Superoxide anion release in the retinal pigment epithelium (RPE) was analyzed by chemiluminescence. Retinal morphology was studied by transmission electron microscopy.

RESULTS. The high-cholesterol group, with increased retinal oxidative stress (P < 0.01) and NO metabolites in the retina (P < 0.05), had increased superoxide anion release (P < 0.05) and showed development of pyknosis, irregular nuclear membranes, and cytoplasmic accumulation of lipids and apoptotic vacuoles in the RPE cells. Vitamins C and E prevented biochemical changes and most ultrastructural alterations in the RPE.

CONCLUSIONS. The results suggest an evolving role for hypercholesterolemia through increased retinal oxidative stress and NO synthesis that could be responsible for retinal ultrastructural alterations. The beneficial effects of vitamins C and E in the retina further support this hypothesis. (Invest Ophthalmol Vis Sci. 2005;46:1140–1146) DOI:10.1167/iovs.04-0516

Age-related macular degeneration (ARMD) is the most common cause of severe and irreversible blindness in people older than 65 years in western Europe and the United States.¹ Because advanced age is the universally accepted risk factor for ARMD,² it is important to understand how age-related changes in Bruch’s membrane (BrM) predispose some individuals to disease. Studies in existing animal models that attempt to simulate ARMD through phototoxicity, acceleration of senescence, candidate gene manipulation, and high-fat diets do not fully replicate the clinical, histologic, and angiographic features of human disease, probably because of the multifactorial nature of ARMD.¹ Hypercholesterolemia and a high-fat diet also have been proposed as possible risk factors for retinal diseases such as ARMD.³,⁴ Murine studies have shown that dietetic or genetic hypercholesterolemia induce retinal changes similar to those found in the aged human retina.⁵–⁸

Dietary fat and cholesterol have been linked to an increased incidence of coronary heart disease, and lipids are thought to contribute to vascular injury. Oxidized low-density lipoprotein (LDL) especially stimulates inflammation and favors atherosclerosis by promoting cytokine production.⁹,¹⁰ Moreover, recent evidence suggests that abnormal lipid levels may contribute to the development of ARMD, either directly or by promoting vascular disease.¹¹,¹² Therefore, it is plausible that the progression of ARMD and atherosclerotic disease may share similar pathogenic mechanisms.¹² Curcio et al.¹³ reported data that strengthen the rationale for seeking links between these two diseases at the tissue, cellular, and molecular levels,¹³ but atherosclerosis and ARMD are complex multifactorial diseases with many environmental and genetic factors contributing to the final outcome. The role of cardiovascular disease and its risk factors in the development of ARMD is unclear, and most of the epidemiologic data to date have been inconsistent regarding this relationship.¹⁴ Increased blood pressure and atherosclerosis, by virtue of their effects on the choroidal circulation and lipid deposition in BrM (i.e., reduced permeability), may increase the risk of development of ARMD.¹⁵ The mechanism by which hypercholesterolemia is involved in pathologic processes such as atherosclerosis could be related to the increase in tissue and systemic oxidative stress, circumstances in which nitric oxide (NO) can play a deleterious role through metabolites such as peroxynitrite. Oxidation and nitrative stress can modify DNA, proteins, and lipids, and extensive data suggest that oxidative damage may play a major causal role in several human diseases.¹⁶ Lipid peroxidation and NO also are thought to be etiopathogenetic factors in retinal processes such as ARMD.¹⁷ The goals of the present study were to determine whether systemic increases in oxidative stress and changes in NO synthesis observed in porcine hypercholesterolemia also are evident in vitreoretinal tissue and to identify ultrastructural alterations in the retinal pigment epithelium (RPE) associated with this hypercholesterolemic profile.

In addition, studies in different animal models have considered the potential for different dietary antioxidants to help prevent development and progression of atherosclerosis¹⁸–²⁰ and retinal diseases.²¹ In contrast to the lack of beneficial effects of antioxidant vitamins on a reduction of atherosclerotic risk, recent clinical studies have shown that antioxidant treatment could stop progression of some of these retinal processes.²²,²³ For that reason, we also studied the effect of vitamins C and E on the ocular biochemical and structural
parameters evaluated in this porcine model of hypercholesterolemia.

**Materials and Methods**

**Animal Model**

Forty-seven 3-month-old male Yucatan miniature pigs, procured from our breeding center, were housed in a temperature controlled room (20–22°C) with a 12-hour light–dark cycle. The animals were divided into three groups and fed different diets for 8 weeks: the normal-cholesterol (NC) control group (n = 21) received standard porcine Chow (Porcsanders; Sanders, Pamplona, Spain); the high-cholesterol (HC) group (n = 14) received chow containing 24.5% animal lard, 4% cholesterol (Roig Farma; Barcelona, Spain), and 1.5% biliary extract (Roig Farma); and the high-cholesterol plus vitamins C and E (HCV) group (n = 12) received the same chow as the HC group for 3 weeks and thereafter supplementation (up to 8 weeks) with 1 g vitamin C and 1000 IU d,l-a-tocopherol acetate (Roig Farma) per animal per day. This hypercholesterolemic diet has been shown to induce advanced atherosclerotic lesions in coronary arteries of miniature pigs. Dosages of vitamins were chosen from similar models of porcine hypercholesterolemia demonstrating that pigs treated with vitamins C and E have smaller degree of LDL oxidation, preserved endothelial function, and best response to vascular injury. The beginning of the diets was scheduled in such a way that the length of the treatments was the same for all the animals, and no more than two pigs were killed each day.

The animals were fasted overnight before the day of death but were allowed access to water ad libitum and were anesthetized by an intravenous (IV) injection of ketamine (10 mg/kg; Ketolar; Parke Davis, Switzerland). LDL was calculated using the formula of Friedewald.

**Lipid Plasma Analysis and Determination of Vitamin E Content**

Total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL) were measured in plasma by an enzymatic method by a fully automated clinical analyzer (Hitachi 717; Roche Diagnostics, Basel, Switzerland). LDL was calculated using the formula of Friedewald et al., which was modified for the ratio of cholesterol to TG in very-low-density porcine lipoproteins (8:1). The tocopherol concentration in plasma was measured by high-performance liquid chromatography.

**Lipid Peroxidation in Plasma, Vitreous, and Retinal Homogenate Based on TBARS Measurement**

Thiobarbituric acid reactive substances (TBARS) were measured in plasma, retinal, and vitreous homogenates, as an indicator of lipid peroxidation.

**Determination of Superoxide Production in RPE**

The generation of superoxide from the porcine posterior pole was estimated using lucigenin-enhanced chemiluminescence, as described previously. Scintillation vials containing 2 mL PBS buffer with 5 μM lucigenin were placed in a scintillation counter switched to the out-of-coincidence mode. After 15 minutes, background counts were recorded, and an optic cup was then added to the vial. Scintillation counts were recorded every minute for 40 minutes and the respective background counts subtracted. The results are expressed as the average counts per minute per gram of tissue.

**Quantification of Total NOx Concentration in Retinal and Vitreous Homogenates**

Total NO metabolites (NOx), nitrite (NO2⁻), nitrate (NO3⁻), N-nitrosodiazines, nitrosyl hemoglobin adducts, and N-nitrosoamines, were determined with an NO chemiluminescence analyzer (NOA 280; Sievers Instruments, Boulder, CO). Briefly, the samples were injected into a reaction chamber containing vanadium chloride in an HCl (2 M) solution that was boiled (90°C under weak vacuum) and bubbled with helium gas. This mixture reduces NOx to NO, which then is measured by reaction with ozone in the chemiluminometer.

The results were corrected for background levels of NO present in saline alone and by protein content determined by the method of Bradford and were expressed as nanomoles NO per milligram.

**Electron Microscopy**

Three left eyes from each group were processed for histologic examination. Whole enucleated porcine eyes were fixed in 2.5% glutaraldehyde, 0.1 M cacodylate, and 0.2 M PBS. The posterior pole was dissected as described previously and postfixed in 1% osmium tetroxide, stained with 1% uranyl acetate, and embedded in Epon Araldite resin. One-micrometer sections were cut with an ultramicrotome, stained with 2% toluidine blue O, and examined under a light microscope to determine the areas of interest. Thin sections (~50–90 nm) were cut, collected on copper grids, and stained with 4% uranyl acetate and lead citrate. Subsequently, three sections from each animal were evaluated by transmission electron microscopy (TEM; EM10; Carl Zeiss, Thornwood, NY) and were photographed for posterior analysis.

**Statistical Analysis**

All data are presented as the mean ± SEM. Analysis of variance (ANOVA) or the Kruskal-Wallis test was applied to assess differences between treatment groups. After a significant ANOVA or Kruskal-Wallis result was found, comparisons were made with the Bonferroni post hoc test or Mann-Whitney test, respectively. The Wilcoxon rank test was used to evaluate paired comparisons. Associations between variables were assessed using Spearman’s correlation test. P < 0.05 was considered statistically significant, and analysis was performed on computer (Sigma Stat software; SPSS Inc., Chicago, IL).

**Results**

**Plasma Lipids after Hypercholesterolemic Diet and Vitamin E Levels after Antioxidant Supplementation**

Baseline concentrations of TC, HDL, and LDL were similar in all groups. The hypercholesterolemic diet significantly increased (up to 250%; P < 0.01) the TC levels in the HC group and also resulted in higher plasma lipid peroxidation compared with the control (P < 0.01) at the time of death (Table 1). The increase in the TC levels was attributed primarily to elevated LDL levels (P < 0.01), although the HDL levels also were...
TABLE 1. Plasma TC, HDL, LDL, TG, TBARS, and α-Tocopherol at Baseline and at the End of the Experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>TC</th>
<th>LDL</th>
<th>HDL</th>
<th>TG</th>
<th>TBARS</th>
<th>Tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal values</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>61.50 ± 4.25</td>
<td>24.22 ± 3.09</td>
<td>35.60 ± 1.95</td>
<td>43.15 ± 3.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>58.50 ± 1.93</td>
<td>21.80 ± 2.30</td>
<td>33.62 ± 1.89</td>
<td>44.98 ± 3.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td>57.38 ± 2.32</td>
<td>20.59 ± 1.16</td>
<td>36.00 ± 1.92</td>
<td>48.65 ± 2.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At death</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>65.50 ± 2.84</td>
<td>24.10 ± 2.16</td>
<td>31.70 ± 1.44</td>
<td>45.60 ± 5.42</td>
<td>0.744 ± 0.067</td>
<td>2.00 ± 0.41</td>
</tr>
<tr>
<td>HC</td>
<td>182.00 ± 22.67*</td>
<td>126.50 ± 20.83*</td>
<td>46.80 ± 3.50*</td>
<td>40.80 ± 8.20</td>
<td>1.458 ± 0.306*</td>
<td>1.67 ± 0.22</td>
</tr>
<tr>
<td>HCV</td>
<td>188.10 ± 29.91*</td>
<td>119.90 ± 25.64*</td>
<td>61.90 ± 6.88†</td>
<td>32.10 ± 5.25</td>
<td>0.635 ± 0.090†</td>
<td>14.04 ± 3.66†</td>
</tr>
</tbody>
</table>

Table data are expressed as mean milligrams per deciliter (plasma TC, LDL, HDL, TG), equivalent MDA (TBARS), and millimoles/L (α-tocopherol) ± SEM. Statistically significant differences from the control group are indicated as *P < 0.01, or †P < 0.05 and ‡P < 0.01.

Changes in Ocular Lipid Peroxidation Induced by Hypercholesterolemia and the Effect of Vitamin C and E Supplementation

Induction of oxidative stress by hypercholesterolemia was assessed by measuring the formation of adducts with thiobarbituric acid. Retinal homogenates from the HC group showed a significant increase in TBARS compared with the control group (P < 0.001; Table 2). TBARS correlated positively with plasma TC (r = 0.57, P < 0.01; Fig. 1A) and LDL (r = 0.64, P < 0.001; Fig. 1B) in the HC group. However, the TBARS concentration in retinal homogenates from the vitamin-treated hypercholesterolemic pigs (HCV group) was significantly lower than in the HC group (P < 0.001; Table 2), although it was still higher than in the control group (P < 0.05). Retinal TBARS in the HCV group correlated negatively with circulating TC (r = −0.56, P < 0.05; Fig. 1C) and LDL (r = −0.82, P < 0.001; Fig. 1D). Lipid peroxidation in vitreous homogenates was similar in the three experimental groups (Table 2).

Hypercholesterolemia-Derived Superoxide Anion and the Effect of Antioxidant Treatment

To determine whether the observed differences in lipid peroxidation are associated with changes in free radical synthesis, superoxide anion production was measured in the RPE by performing lucigenin-enhanced chemiluminescence.

Hypercholesterolemia induced an increase in superoxide anion production compared with control animals (P < 0.05; Table 2), whereas hypercholesterolemic animals that received vitamins C and E had significantly lower superoxide anion synthesis similar to the control animals (P < 0.001; Table 2). Furthermore, the superoxide anion increase induced by hypercholesterolemia correlated positively with TC in plasma (r = 0.53, P < 0.05; Fig. 2A) and retinal lipid peroxidation (r = 0.50, P < 0.05; Fig. 2B).

NOx Metabolites in Hypercholesterolemia and the Effect of Vitamins

Having demonstrated that vitamins C and E prevent increased lipid peroxidation and superoxide anion production in hypercholesterolemic animals, we then measured tissue NO synthesis in this animal model. Total NO production, as determined by NOx content, increased substantially in retinal and vitreous homogenates from the HC group (P < 0.05; Table 2). Vitamins C and E reduced total NOx production in the retina and vitreous compared with the HC group (P < 0.01 and P < 0.05, respectively) and reached concentrations similar to those in the control group. The retinal NOx content correlated positively with RPE superoxide anion production (r = 0.81, P < 0.001; Fig. 2C).

Ultrastructural Hypercholesterolemic Alterations in the RPE and BrM and the Effect of Antioxidant Supplementation

The morphology of the posterior pole was analyzed by TEM to determine whether the observed biochemical changes were associated with morphologic alterations. Eyes from hypercholesterolemic animals exhibited ultrastructural features that were different from the control group, such as nuclear and cytoplasmic alterations, together with discontinuities in BrM. The most important finding was the presence of RPE cells with an irregular nuclear membrane and pyknotic nuclei (Fig. 3A), which was in marked contrast with the smooth rounded nuclei present in RPE cells of the control animals (Fig. 4A). Moreover, BrM showed frequent ruptures localized in its elastic lamina (Fig. 3B). Lipid and autophagocytic vacuoles (250–500 nm in

Table 2. Results of Retinal and Vitreous Lipid Peroxidation and NOx and Superoxide Radical Production in the RPE

<table>
<thead>
<tr>
<th>Lipid Peroxidation (MDA)</th>
<th>NO Metabolites</th>
<th>Superoxide Anion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitreous Retina</td>
<td>Vitreous Retina</td>
<td>RPE</td>
</tr>
<tr>
<td>NC (n = 21)</td>
<td>1.18 ± 0.31</td>
<td>5.41 ± 1.35</td>
</tr>
<tr>
<td>HC (n = 14)</td>
<td>1.37 ± 0.30</td>
<td>22.00 ± 3.82***</td>
</tr>
<tr>
<td>HCV (n = 12)</td>
<td>0.40 ± 0.09</td>
<td>9.49 ± 2.29**†</td>
</tr>
</tbody>
</table>

Values are expressed as mean nanomoles per milligram of protein (MDA and NO metabolites) and counts per minute per milligram of weight (superoxide radical production) ± SEM. Statistically significant differences from the control group are indicated as *P < 0.05, **P < 0.01, or ***P < 0.001, and between HC and HCV as †P < 0.05, ††P < 0.01, and †††P < 0.001.
diameter; Fig. 3B) also were observed in RPE cells in the HC
group, accompanied by an increase in the number of autophagosomes (Fig. 3C), whereas these organelles were absent in the
RPE cells of the control pigs. Vitamin supplementation pre-
vented the development of nuclear alterations in RPE cells and
reduced the number of lipid vacuoles and autophagosomes
(Fig. 4B). No drusen or neovascularization was observed in any
of the sections.

DISCUSSION

The present study is the first to demonstrate increased oxida-
tive stress and NO production in the neurosensory retina in a
porcine model of hypercholesterolemia, thus establishing an
association with ultrastructural alterations in the RPE. The fact
that vitamins C and E prevented biochemical and morphologic
changes strongly suggests that hypercholesterolemia-derived
oxidative stress is responsible for the observed alterations.

When generation of reactive oxygen species exceeds the
antioxidant capacity, oxidative stress results, which is believed
to have an important mechanistic role in the pathogenesis of
several inflammatory and degenerative diseases, including ather-
sclerosis, diabetes mellitus, and ARMD.25,29 The effect of a
high-fat diet and hypercholesterolemia through increased oxida-
tive stress is well documented in the vascular system and
other tissues,30 and has been proposed as a risk factor in
human ophthalmic diseases such as ARMD.5,17,31 Elevated met-
bolic activity, exposure to direct light, and the high polyunsat-
saturated fatty acid (PUFA) content of photoreceptor outer
segments expose the retina to an increased risk of lipid per-
oxidation by unopposed action of free radicals, and this sus-
ceptibility increases with age in the macular region.32 How-
ever, only a few studies have assessed the effect of
hypercholesterolemia in clinical or experimental models of
retinal diseases.

We studied a porcine model of hypercholesterolemia with
plasma total and LDL cholesterol concentrations that were
closer to humans than those in other experimental models of
hypercholesterolemia, such as rabbits or genetically modified
mice. In our study hypercholesterolemia induced a marked
increase in retinal lipid peroxidation, besides the expected
increase in systemic oxidative stress, assessed as TBARS in

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932933/)

**Figure 1.** Positive correlation among TBARS in retina and plasmatic cholesterol (A) and LDL (B) in the HC group and a negative correlation (C, D) among those parameters in the HCV group. MDA: malondialdehyde.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932933/)

**Figure 2.** Positive correlation among superoxide anion production in the RPE and plasmatic cholesterol (A), TBARS in the retina (B), and retinal NOx (C) synthesis in the HC group.
plasma. Even though increased retinal TBARS also were described in other experimental models of retinopathy (i.e., the diabetic rat), no studies have reported this observation in hypercholesterolemia.

Because retinal oxidative stress results from an imbalance between the production of and defense against free radical species in the retina, we assessed superoxide anion production in the RPE by lucigenin chemiluminescence and showed that production increases substantially in hypercholesterolemic animals. The positive correlation between plasma TC, RPE superoxide, and retinal TBARS in the HC group leads us to propose that hypercholesterolemia induces lipid peroxidation in the eye at least by an increase in superoxide anion production. Xanthine and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases should be considered among the potential sources of oxidative damage in the retina. Xanthine oxidase has been found in the cones and the capillary endothelial cells of the inner retina, whereas NADPH oxidase activity increased in the retinas of diabetic rats. Superoxide dismutase and functionally coupled catalase constitute the main physiologic scavenging system for this free radical, and both enzymes are functionally present in the RPE. Further studies analyzing the effect of hypercholesterolemia on the retinal balance between the expression of antioxidant (i.e., superoxide dismutase, catalase, glutathione peroxidase) and pro-oxidant (i.e., NADPH oxidase, xanthine oxidase) genes are needed to characterize our proposed mechanism properly.

Aside from its own deleterious effects or the possible reduction to hydrogen peroxide, superoxide is of particular interest because it can react with NO to produce the highly reactive peroxynitrite, which can result in cytotoxicity due to lipid peroxidation, inactivation of enzymes by oxidation of protein sulfhydryls and nitration of tyrosines, and damage to DNA and mitochondria. We found that hypercholesterolemia induces a marked increase in vitreoretinal concentration of NO metabolites. NO is constitutively synthesized in the eye to exert its main physiologic functions: maintenance of vascular tone, regulation of vascular permeability, and neuromodulation of synaptic transmission. However, increased NO production from its inducible synthase (iNOS) occurs in different cell types in the eye associated with pathologic conditions such as retinal degeneration, ocular inflammation, cataracts, and diabetes. Thus, in conditions of increased oxidative stress, excess superoxide radicals decrease NO bioavailability through peroxynitrite formation and may inhibit the regulatory effects of NO on systemic and ocular blood flow and visual transduction. We speculate that iNOS is responsible for the observed NO increase, because it is induced by oxidative stress and inflamma-

**Figure 3.** Transmission electron micrograph of the outer retina and choroid of hypercholesterolemic animals. (A) In RPE cells, three nuclei were visible with nuclear alterations such as nuclear pyknosis and irregular membrane (white arrows) near a normal, round nucleus (N). (B) Moreover, cytoplasmic autophagocytic vacuoles filled with electron dense material (black arrow) and empty (✱) were present. BrM revealed frequent breaks in the elastic lamina (white arrow). (C) RPE cytoplasm revealed an increase in the number of autophagosomes (✱). Scale bars, 5 μm.

**Figure 4.** (A) Electron micrographs showing normal RPE morphology in the control group. BrM appeared normal and nuclei (N) observed were rounded and with no pyknosis. (B) Hypercholesterolemic animals supplemented with vitamins C and E had no nuclear alterations and marginal cytoplasmic vacuoles (✱). N, nucleus. Scale bars, 5 μm.
ory cytokines. However, further experiments are needed to identify positively the NOS isoforms responsible for increased NO synthesis, together with their cellular localization within the RPE and/or retina.

The increased production of superoxide anion in hypercholesterolemic RPE with higher NOx concentration, as indicated by their positive correlation, suggests lower NO bioavailability for exerting its physiologic actions and a higher risk of tissue damage by increased production of peroxynitrites. This point should be further confirmed in future studies by measuring nitrotyrosine residues in the RPE and retina, since peroxynitrites may attack tyrosine residues in protein to generate nitrotyrosine, which may serve as an in vivo biomarker for nitrative stress.

Our observations, novel in hypercholesterolemia, agree with the increased retinal nitrate and oxidative stress found in experimental models of diabetic retinopathy. Furthermore, the interaction between NO and superoxide was also related to an increased risk of failed choroidal perfusion and lipid peroxidation, two mechanisms involved in the development of ARMD.

Although our study did not establish a causal relationship, we found several cytoplasmic and nuclear ultrastructural alterations in the RPE of hypercholesterolemic pigs associated with the aforementioned biochemical changes. We found an increase in the number and size of lipid-like droplets and autophagocytic vacuoles in the cytoplasm and pyknotic nuclei with irregular membranes that could lead to RPE degeneration. Considering that oxidative stress affects the photoreceptors, we hypothesize that the phagocytic vacuoles could result from the increased catabolism of photoreceptors outer segments. The observed alterations are similar to those found in a fat-fed atheregenous mouse model. It is widely believed that a defective RPE is the underlying cause of human retinal and macular diseases and dystrophies, because the first observed clinical changes in ARMD seem to occur in the RPE. Furthermore, our observations support the conclusions of human studies that connect cholesterol and saturated fat intake with ARMD.

To assess further whether oxidative stress is involved in the biochemical and ultrastructural alterations observed in vitreoretinal tissue and RPE, we studied the effect of vitamins C and E on these ocular parameters. These treatments were chosen from similar models of porcine hypercholesterolemia that showed the two vitamins prevent LDL oxidation and preserve vascular function. Hypercholesterolemic pigs given vitamins C and E supplementation had increased plasma tocopherol and reduced plasma TBARS without changes in cholesterol levels and exhibited a significantly less retinal lipid peroxidation. The fact that vitamin treatment reduced systemic lipid peroxidation to control levels while retinal TBARS remained higher than in the NC group could reflect ocular exposure to a higher risk of oxidative stress. We found an unexpected negative correlation between retinal lipid peroxidation and plasmatic levels of cholesterol in the HCV group, which could be the result of the positive correlation between tocopherol and cholesterol levels in that group.

The observation of markedly reduced production of superoxide anion in the RPE of vitamin-supplemented hypercholesterolemic animals, even below that of the control animals, leads us to believe that this is at least one of the mechanisms responsible for the decreased oxidative stress in the retina. Moreover, we also found a lower NOx concentration in the retina and vitreous of the HCV group, reaching levels similar to those in the control animals. Decreased synthesis of superoxide and NO could translate into a markedly reduced likelihood of peroxynitrite generation, which also could account for decreased lipid peroxidation, although this should be confirmed further by demonstrating the peroxynitrite reduction in the retina and/or RPE by vitamins C and E.

Although vitamin E can be a free radical scavenger, it also acts as a chain-breaking antioxidant in lipoproteins and biological membranes, due to its lipid solubility, preventing lipid peroxidation of PUFA and modification of proteins by reactive oxygen species. Vitamin C allows α-tocopherol regeneration from α-tocopheroxy radicals, thereby preventing vitamin E pro-oxidant activity and tocopherol-mediated peroxidation, besides scavenging hypochlorous acid and the tyrosyl radical and protecting lipids from reactive oxygen and nitrogen species. Aside from the direct effects, vitamin E inhibits NADPH oxidase-dependent generation of superoxide through decreased protein kinase C (PKC) activity.

Vitamins C and E not only prevented biochemical changes in the retina but also drastically reduced the severity of ultrastructural alterations. HCV animals had normal nuclei, similar to those in the NC group, and a small number of phagocytic vacuoles. Our results confirm previous reports on the beneficial effect of vitamin E in a much more extreme model, the light-damaged mouse with combined genetic and long-term dietetic hypercholesterolemia. Moreover, vitamin E supplementation in hypercholesterolemic rats induced a significantly decrease in lipid peroxide concentrations and a significant increase in the glutathione content and antioxidant activities in erythrocytes and liver. In conclusion, our results support an evolving role for hypercholesterolemia in the development of ultrastructural RPE changes through increased vitreoretinal oxidative stress and NO synthesis. Our data also suggest that vitamins C and E may have a beneficial effect on the prevention and/or early treatment of hypercholesterolemia-related ocular changes. Although our porcine model exhibits lipid metabolism abnormalities similar to those observed in human hypercholesterolemia, differences in endogenous antioxidant systems and in the development of retinal alterations may exist between pigs and humans. Therefore, further studies are needed to confirm the role of hypercholesterolemia and oxidative stress in human retinal alterations.

Acknowledgments

The authors thank María Antonia Eleta (Department of Histology and Pathology, University of Navarra) for technical assistance in electron microscopy.

References