Age-related macular degeneration (AMD) is a slow, progressive disease with both genetic and environmental risk factors. Free radical-induced oxidation of docosahexaenoate (DHA)-containing lipids generates ω-(2-carboxyethyl)pyrrole (CEP) protein adducts that are more abundant in ocular tissues from AMD than normal human donors. To understand better the role of oxidative damage in AMD, we have synthesized CEP-modified proteins, produced anti-CEP antibodies, and initiated analysis of CEP immunoreactivity and autoantibodies in human plasma. A highly selective rabbit polyclonal anti-CEP antibody was raised that binds CEP 1000 times more strongly than carboxypropylpyrrole, a close structural analogue. The CEP adduct uniquely indicates oxidative modification from DHA derivatives because CEP protein modifications cannot arise from any other common polyunsaturated fatty acid. Immunocytochemistry localized CEP to photoreceptor rod outer segments and retinal pigment epithelium in mouse retina and demonstrated more intense CEP immunoreactivity in photoreceptors from a human AMD donor compared with healthy human retina. The mean level of anti-CEP immunoreactivity in AMD human plasma (n = 19 donors) was 1.5-fold higher (p = 0.004) than in age-matched controls (n = 19 donors). Sera from AMD patients demonstrated mean titers of anti-CEP autoantibody 2.3-fold higher than controls (p = 0.02). Of individuals (n = 13) exhibiting both antigen and autoantibody levels above the mean for non-AMD controls, 92% had AMD. These results suggest that together CEP immunoreactivity and autoantibody titer may have diagnostic utility in predicting AMD susceptibility.
Polyunsaturated Fatty Acid

Hydroxy-ω-oxoalkenoic Acid

Carboxyalkylpyrrole

Fig. 1. Generation of 2-(ω-carboxyalkyl)pyrrole epitopes. Oxidative fragmentation of polyunsaturated fatty acids generates a host of oxidation products, including the hydroxy-ω-oxoalkenoic acids HODA, HOOA, and HOA, which give rise to a host of protein modifications, including the family of carboxyalkylpyrrole protein adducts CHP, CPP, and CEP.

Experimental Procedures

Materials—Chicken egg ovalbumin (grade V), bovine serum albumin (BSA, fraction V), human serum albumin (HSA, fraction V), and arachidonic acid were purchased from Sigma; the dipeptide Ac-Gly-Lys-OMe was purchased from Bachem, Torrance, CA; and keyhole limpet hemocyanin (KLH) was purchased from Calbiochem. All organic chemicals including the family of carboxyalkylpyrrole protein adducts CHP, CPP, and CEP.

Here we explore further the role of oxidative fragmentation of DHA in AMD. We report methods for preparing CEP-modified proteins and characterization of a rabbit polyclonal anti-CEP antibody. In addition, we present evidence that CEP identifies proteins and characterization of a rabbit polyclonal antibody, which can generate peptide-bound CEP adducts. Although rare in most human tissues, DHA is present in ~80 mol % of the polyunsaturated lipids in photoreceptor outer segments (13). The abundance of DHA in photoreceptors, the high phototoxic stress in retina, and the fact that DHA is the most oxidizable fatty acid in humans (13) suggests that DHA oxidation products may have possible utility as biomarkers for AMD susceptibility.

Human Plasma Preparation—Blood was collected into 7-ml vacutubes containing EDTA (10.5 mg), from AMD and normal healthy donors at the Cole Eye Institute, Cleveland Clinic Foundation. Cells were removed by centrifugation at 2500 rpm (1300 × g) for 10 min, and the plasma was transferred to plastic vials containing butylated hydroxytoluene (BHT; 1 mg/ml), leupeptin (35 μM), pepstatin (5 μM), and aprotinin (0.1 trypsin inhibitor unit/ml). Plasma was then quick-frozen in liquid nitrogen and stored at -80 °C until used. Usually, BHT and protease inhibitors were added to the plasma samples 3–5 h after blood was drawn.

Preparation of ω-Carboxyethylpyrrole-modified Peptide and Proteins—Paul-Knorr reactions of ω-carboxyaryl compounds with primary amines provided an efficient route to 2-ω-carboxyalkylpyrroles (7). Syntheses of ω-ketoaldehydes, 4,7-dioxoheptanoic acid (DOHA), and its phosphatidylcholine ester (DOHA-PC) were developed as described in the Supplemental Material to allow unambiguous production of 2-ω-carboxyalkylpyrrole (CEP) and the corresponding PC ester. First, a Grignard reagent was produced from 2-(2-bromoethyl)-1,3-dioxolane, followed by selective acylation of this organomagnesium derivative with 3-carboxypropionyl chloride. Saponification of the resulting ethylene ketal ester delivered a carboxylic acid that, when coupled with 2-lysophosphatidylcholine, gave a stable precursor that yielded DOHA-PC upon hydrolysis. Transketolization of the ethylene ketal with acetone provided a mixture containing DOHA, presumably in equilibrium with the corresponding hemiacetal based on the 1H NMR spectrum that exhibits two aldehyde hydrogen singlets (Supplemental Fig. S5). Paul-Knorr condensation of DOHA or DOHA-PC with the dipeptide acetyl-Gly-Lys-O-methyl ester generated the CEP-dipeptides, DOHA-dipeptide, or CEP-PC-dipeptide that were fully characterized by 1H NMR (12). 1H NMR and 13C NMR spectra were recorded at 300 and 75 MHz, respectively, and solvents and chromatography conditions for purification of synthetic products were as described elsewhere (12). All reactions in an inert atmosphere were under argon unless otherwise specified. As described in detail in the Supplemental Material, Paul-Knorr condensation with DOHA was used to prepare CEP-modified keyhole limpet hemocyanin (CEP-KLH), bovine serum albumin (CEP-BSA), human serum albumin (CEP-HSA), and glycodehydrolase-3-phosphate dehydrogenase (CEP-GPDH). Protein concentrations were determined using the Pierce bicinchoninic acid protein assay (14), and pyrrole concentrations were determined using the Ehrlich reagent, 4-dimethylaminobenzaldehyde (15), and the above CEP-dipeptide as a reference standard.

Identification of CEP-modified Residues in HSA—CEP-HSA was reduced in 400 mM ammonium bicarbonate containing 8 mM urea and 10 mM dithiothreitol for 30 min at room temperature under argon and then thiol groups were alkylated by the addition of iodoacetamide (to 50 mM). Following alkylation the preparation was dialyzed overnight against 10
and White rabbit was inoculated subcutaneously along the back and washed 3 times with 0.1% ovalbumin plus 0.05% Tween 20 (300 solution was washed away with 0.1% ovalbumin in PBS (300 goat anti-human IgG or alkaline phosphatase-conjugated goat anti-

Secondary antibodies were added (alkaline phosphatase-conjugated goat anti-human IgG, 100 l, and after 30 min the plate was washed with 0.05% Tween 20 containing 0.1% ovalbumin (3 times, 300 l), and then substrate was added (p-nitrophenyl phosphate, 100 l/well, 0.9 mg/ml, pH 9.6, Bio-

Rad). After 60 min, the absorbance was read at 405 nm with reference at 650 nm. The titer was defined as the ratio of plasma binding to antigen (A) versus binding to BSA (A0).

ELISA—Three ELISA procedures were employed and are termed here as Methods A–C (7, 19). Method A was used to determine the titer of anti-CEP antisera. Method B was used to characterize cross-reactivities and to quantify carboxyalkyl pyrrole immunoreactivities. Method C was used to titer anti-CEP autoantibodies in human plasma. ELISA Method A—Anti-CEP antibody titer in rabbit serum was measured with goat anti-carboxyalkyl pyrrole antibodies (7) following the modifications. Baxter 96-well ELISA plates were coated with CEP-HSA containing 1.5 mol of pyrrole per mol of protein by incubating a 4.3 l/g protein solution in PBS (100 l/well) at 37 °C for 1 h in a moist chamber. The coating solution was washed away with PBS (3 x 300 l), and the wells were incubated with blocking solution (1.0% chicken egg ovalbumin in PBS, 300 l) at 37 °C for 1 h. The blocking solution was washed away with 0.1% ovalbumin in PBS (300 l), and then various dilutions of rabbit serum were added (100 l) in PBS containing 0.2% ovalbumin. Ovalbumin (0.2%) was used in PBS without serum and nonimmune rabbit serum, diluted as above, were employed as controls. Following incubation at 37 °C for 1 h, the wells were washed 3 times with PBS containing 0.1% ovalbumin and then washed at 37 °C with 100 l of alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000 dilution). The wells were again washed 3 times with PBS containing 0.1% ovalbumin, and as substrate p-nitrophenyl phospho-

The titer was defined as the ratio of plasma binding to antigen (A) versus binding to BSA (A0).

CEP Biomarkers for AMD

CEP Immunoreactivity from the Reaction of HOHA-PC with HSA—HOHA-PC (6.5 mg, 0.25 mmol) and 4-hydroxy-7-oxodeca-5,9-enone acid ester of 2-lyso phosphatidylycholine (HOHA-PC, 5 mg, 7.9 μmol) (12) were dissolved in PBS (5 ml), yielding a final concentration of 1.6 mM HOHA-PC and 0.05 mM HSA. The preparation was incubated at 37 °C under argon, and aliquots were withdrawn with time. A portion of each aliquot was hydrolyzed by 0.2 N KOH at 37 °C for 30 min, neutralized with HCI, and then dialyzed against PBS (2 x 1 liter) at room temperature for 24 h. Levels of CEP immunoreactivity were determined by ELISA Method B using anti-CEP antibodies.

In Vitro Oxidation of PUFAs or DHA-PC with HSA—Docosahexae-

nic acid (DHA, 6.1 μmol), arachidonic acid (6.6 μmol), or linoleic acid (7.1 μmol) and HSA (0.5 μmol) were dissolved in PBS (10 ml), and incubated with chloroform:methanol (20:1) for 20 min, dried under nitrogen, and 0.8 mM FeSO4 (510 μl). The preparations were incubated up to 72 h at 37 °C under air and aliquots were removed periodically; afterward the oxidation reactions were quenched by the addition of EDTA (to 1 mg/ml) and BHT (to 40 mM) followed by overnight dialysis at room temperature against PBS (2 x 1 liter) containing EDTA (1 mg/ml). Prior to oxidation of DHA-PC, DHA-PC liposomes were prepared (20). Briefly, a solution of DHA-PC (6.3 μmol) in chloroform was evaporated in vacuo, and the resulting lipid film was hydrated in PBS (2 ml) at 37 °C and the suspension extruded through a polycarbonate membrane (100-nm pore size, Nuclepore Co., Pleasanton, CA) to produce a clear solution of unilamellar liposomes. The resulting DHA-PC liposomes were combined with either carboxyalkyl pyrrole antibodies (7) or anti-carboxyalkyl pyrrole antibodies (7) with the following modifications. Baxter 96-well ELISA plates were coated with CEP-HSA containing 1.5 mol of pyrrole per mol of protein by incubating a 4.3 l/g protein solution in PBS (100 l/well) at 37 °C for 1 h in a moist chamber. The coating solution was washed away with PBS (3 x 300 l), and the wells were incubated with blocking solution (1.0% chicken egg ovalbumin in PBS, 300 l) at 37 °C for 1 h. The blocking solution was washed away with 0.1% ovalbumin in PBS (300 l), and then various dilutions of rabbit serum were added (100 l) in PBS containing 0.2% ovalbumin. Ovalbumin (0.2%) was used in PBS without serum and nonimmune rabbit serum, diluted as above, were employed as controls. Following incubation at 37 °C for 1 h, the wells were washed 3 times with PBS containing 0.1% ovalbumin, and as substrate p-nitrophenyl phospho-

Cell Preparation—DHA-PC (6.3 mg) was digested at 37 °C with 0.2 N KOH at 37 °C for 30 min, neutralized with HCI, and then dialyzed against PBS (2 x 1 liter) at room temperature for 24 h. Levels of CEP immunoreactivity were determined by ELISA Method B using anti-CEP antibodies.

The results are expressed as Mean ± S.D. of triplicate determinations. For statistical analysis of plasma levels of CEP immuno-

reactivity and autoantibodies, p values were calculated by independent
CEP Biomarkers for AMD

RESULTS

We produced CEP-added proteins using Paal-Knorr condensation of DOHA with the ε-amino group of lysyl residues. The unambiguous synthesis of DOHA, DOHA-phosphatidylcholine (DOHA-PC), CEP, and CEP-PC added dideptide and verifications by NMR are documented in the Supplemental Material. DOHA was then used to chemically modify KLH, BSA, HSA, and GPDH to yield CEP-added antigens and reference proteins.

Characterization of DOHA Modified HSA—To probe for the presence of CEP adducts in HSA following modification with DOHA, the protein was digested with trypsin and analyzed by mass spectrometry (Fig. 2). Two HSA peptides containing apparent lysyl CEP adducts were identified by MALDI-TOF mass spectrometry at m/z 1141 and m/z 2021 (Fig. 2A). Tandem nanoelectrospray mass spectrometric analysis of m/z 1141 revealed HSA peptide 234AK*WAVAR241 and unambiguously confirmed a CEP adduct on lysyl residue Lys236 (Fig. 2B). Electrospray MS/MS analysis of m/z 2021 revealed HSA peptide 170PYFYPHELFFAK*R241 and a CEP adduct on lysyl residue Lys316 (Fig. 2C). The pyrrole content in this preparation of CEP-HSA was estimated using the Ehrlich assay to be 1.5–1.6 mol of pyrrole per mol of protein. Complete structural analysis of the DOHA-modified albumin was not pursued, and other residues containing CEP adducts could be present.

Anti-CEP Antibody Specificity—To evaluate the binding specificities of anti-CEP rabbit polyclonal antibodies raised to DOHA-modified KLH, competitive ELISAs were performed with HSA adducted with CEP, CPP, CHF, or PP-ACA, and the results (Fig. 3 and Table I) were compared with that of anti-CPP antibodies (7). The CEP and CPP adducts differ by only a single CH group; nevertheless, inhibition of anti-CEP antibody binding by CPP-HSA is remarkably weak, i.e. 0.1% cross-reactivity. Inhibition of anti-CPP binding by CEP-HSA is somewhat greater than that for CPP-HSA; however, anti-CEP also exhibits high structural specificity, i.e. only 1.3% cross-reactivity. Neither antibody exhibits significant cross-reactivity with CHF-HSA, which contains a much larger carboxyalkyl group than the haptons against which anti-CEP or anti-CPP were raised. Another pyrrole-containing species, PP-6-ACA-HSA, which contains an n-pentyl rather than a carboxyalkyl side chain, showed no cross-reactivity with either of the antibodies. Similar structural selectivity of anti-CEP antibody was observed when a different coating agent, CEP-GPDH, was used. As expected, unmodified HSA or GPDH were not recognized by anti-CEP antibody.

CEP Immunoreactivity from HOHA-PC Modification of HSA—Recently we showed that 2-(4-hydroxy-7-oxohept-5-enoyl)phosphatidylcholine (HOHA-PC), a minor oxidative product from DHA-PC, can react with the ε-amino group of a lysine-containing dideptide to form CEP adducts (12). HOHA-PC will also modify HSA resulting in anti-CEP immunoreactivity as shown in Fig. 4A; however, the antibody inhibition curves do not parallel that for the CEP-HSA generated by DOHA modification of HSA. Presumably this is because the CEP epitopes generated with HOHA-PC are mostly in the form of PC esters rather than the free acids against which the antibodies were raised. Treatment of the reaction product mixture with KOH converted the PC-esters into the corresponding free acids whose inhibition curves parallel that for CEP-HSA (Fig. 4B). The yield of CEP epitope from modification of HSA with HOHA-PC after 24 h was estimated to be ∼0.5% relative to the phospholipid fragment.

Carboxyalkylpyrrole Immunoreactivity from Oxidation of PUFA or DHA-PC—In vitro free radical-initiated oxidations of arachidonic acid, linoleic acid, or DHA for 72 h in the presence of HSA generated carboxyalkyl pyrrole immunoreactivity. Protein-bound pyroles were detected by ELISA Method B using the appropriate BSA-bound pyrrole as coating agent and the corresponding HSA-bound pyrrole as standard. For CEP and CPP, barely detectable levels of immunoreactivity were observed, namely ∼8 μmol of CEP/mmol of HSA, 7 × 10⁻⁶% yield relative to DHA and ∼5 μmol of CPP/mmol of HSA, 40 × 10⁻⁶% yield relative to arachidonate. A significantly greater level of CHF immunoreactivity was observed, namely ∼2.8 mmol of CHF/mmol of HSA, 0.02% yield relative to linoleate. Oxidation of DHA-PC liposomes for 24 h in the presence of HSA also generated barely detectable CEP immunoreactivity, but following hydrolysis of the PC esters to the corresponding free acids with 0.2% KOH, CEP immunoreactivity increased to ∼50 μmol/mmol HSA or 4 × 10⁻⁴% yield relative to DHA-PC. We speculate that the increase in CEP immunoreactivity following saponification is because of the importance of strong interactions of the free carboxyl group with the anti-CEP antibody, interactions that are not possible when the carboxyl group is esterified to 2-lyso-phosphatidylcholine. A previous study of CEP immunoreactivity generated upon oxidation of low density lipoprotein in vitro also found a large increase consequent to saponification of the oxidized low density lipoprotein (7). The sensitivity of CEP yield to its phospholipid origin is presumably a consequence of interference by the free carboxyl group in HOHA with pyrrole formation. In HOHA-PC, the carboxyl group is masked by esterification. CEP yield from HODA is presumably much greater than CEP yield from HOHA because the carboxyl group in HODA does not interfere with CEP formation because of the longer chain separating it from the γ-hydroxy-α,β-unsaturated aldehyde functionality in HODA (7 carbons) compared with HOHA (2 carbons).

CEP Immunoreactivity in Retina—Immunocytochemical analysis of mouse retina demonstrated intense CEP immunoreactivity in the photoreceptor outer segments and RPE and lighter immunoreactivity in the inner plexiform layer (Fig. 5A). In contrast, when the anti-CEP antibody was preincubated with CEP-protein antigen, all labeling was eliminated. The distribution of CEP immunoreactivity in the RPE and photoreceptor outer segments was confirmed using 9 eyes from three mice with tissue fixation performed shortly after death (<2 h). Mouse retinal tissues were consistently labeled over the photoreceptors and RPE over antibody dilution ranges of 1:100 to 1:10,000. Comparison of anti-CEP immunostaining of human AMD retina with healthy retina (Fig. 5, B and C) show that for the two samples analyzed, more CEP immunoreactivity was present in the AMD tissue than the normal retina. This is concordant with our previous detection of more CEP immunoreactivity by Western analyses of proteins obtained from RPE/Bruch’s membrane/choroid tissue from 11 AMD donors compared with 11 normal controls (6).

CEP Immunoreactivity in Human Plasma—The levels of CEP immunoreactivity were measured by ELISA in human plasma from (i) 19 donors with diagnosed AMD and of average age 82 years, (ii) 19 normal donors of average age 83 years without AMD, and (iii) 9 young healthy donors of average age 27 years. Protease inhibitors, a metal chelator, and an antioxidant were added to human plasma to limit in vitro oxidation/degradation, and all samples were quenched-frozen immediately in liquid nitrogen after plasma isolation (21). A comparison of CEP immunoreactivity detected in each donor group is pre-
Fig. 2. Localization of CEP adducts in DOHA-modified HSA. A, MALDI-TOF mass spectrum of a tryptic digest of CEP-HSA. *Arrows* denote internal standards; *dots* indicate tryptic peptides from HSA; and *asterisks* highlight possible CEP-modified peptides based on a mass addition of 122.0366 for the CEP adduct. *B*, nanoelectrospray MS/MS spectrum of the doubly charged ion \( m/z \) 571 (singly charged \( m/z \) 1141.6302 in A) from HSA residues 234–241 supporting CEP modification of Lys236. *C*, nanoelectrospray MS/MS spectrum of the doubly charged ion \( m/z \) 1101 (singly charged \( m/z \) 2021.0678 in A) from HSA residues 170–184 supporting CEP modification of Lys183.
sent in Fig. 6. Mean CEP immunoreactivity (± S.D.) observed in the plasma of AMD donors (15.9 ± 7.4 pmol/ml) was significantly higher than in the plasma of younger donors (10.5 ± 3.7 pmol/ml, p = 0.05) and also ~1.5-fold higher than in the plasma from older healthy donors (10.5 ± 2.8 pmol/ml, p = 0.004). The mean levels of anti-CEP immunoreactivity measured in triplicate revealed 4–11% intra-assay variability and 3–7% inter-assay variability. The plasma level of CEP adducts is lower than that of CHP adducts (175–275 pmol/ml) (7) and reflects the lower plasma lipid concentration of DHA compared with linoleate, which reflects the lower plasma lipid concentration of DHA compared with linoleate (22).

Anti-CEP Autoantibodies in Human Plasma—Autoantibody titers against CEP protein adducts were measured by ELISA Method C in human plasma from 19 donors with AMD and 19 older normal donors (Fig. 7). Plasma from the AMD donors exhibited a 2-fold higher average CEP autoantibody titer (3.4 ± 3.1 S.D.) than plasma from age-matched normal controls (1.5 ± 0.4 S.D.), and the difference was statistically significant (p = 0.02). Titer here is defined as the ratio of binding to CEP-modified BSA (A) versus unmodified BSA (Ao) and high titer as any AA, value greater than 2 S.D. above the average titer of the controls (namely for these data sets, AA, > 2.3). The prevalence of high anti-CEP autoantibody titers from AMD donors was 53% (10:19) compared with 21% (4:19) of the age-matched controls.

A dot-blot assay was also employed to probe for anti-CEP autoantibodies in which CEP-HSA was immobilized on nitrocellulose membrane, reacted with human plasma and then secondary antibody, and the membrane scanned for spot optical density. Results from the dot blot assay were in good agreement with the ELISA data in Fig. 7 and revealed a mean optical density value (0.15 ± 0.05) for AMD plasma that was 2.5-fold higher (p = 0.01) than the mean optical density value (0.06 ± 0.04) obtained for the age-matched control group (not shown). AMD and age-matched normal plasma exhibiting high levels of anti-CEP antibodies in both assays are highlighted with stars in Fig. 7.

**TABLE I**

| Selectivity of rabbit polyclonal anti-CEP and anti-CPP antibodies |
|---------------------------------------------------------------|
| anti-CEP Antibody | CEP-HSA Coating | CEP-GPDH Coating | anti-CPP Antibody | CEP-BSA Coating |
|-------------------|----------------|----------------|-------------------|----------------|
| IC50 (pmol/well)  | %Cross-reactivity | IC50 (pmol/well) | %Cross-reactivity | IC50 (pmol/well) | %Cross-reactivity |
| HSA CEP-HSA       | 0.02            | 100            | 0.38              | 100            | 12             | 1.3             |
| HSA CPP-HSA       | 19              | 0.1            | 1511              | 0.02           | 0.15           | 100             |
| HSA CHP-HSA       | 24              | 0.1            | 802               | 0.05           | 62             | 0.24            |
| HSA PP-ACA-HSA    | N.D.            | 0              | 4097              | 0.01           | N.D.           | 0               |

The cellular, biochemical, and molecular events contributing to the etiology of AMD remain poorly understood. AMD is usually defined as either “dry” or “wet” and is a slow, progressive degenerative disease with both genetic and environmental risk factors (1, 3). Advanced stage AMD, also known as the wet, exudative, or neovascular form of the disease, affects only ~10% of those with AMD and is characterized by abnormal blood vessel growth from the choriocapillaris through the RPE (neovascularization), resulting in possible hemorrhage, exudation, scarring, and/or serous retinal detachment (23). The vast majority (~90%) of all AMD is the non-neovascular or “dry” form of AMD and is characterized by numerous large macular drusen, atrophy of the RPE, and loss of macular photoreceptors (23). Oxidative damage appears to contribute to the pathogenesis of AMD (4) based on epidemiological studies showing that smoking significantly increases the risk of AMD (1, 24). The molecular mechanism for how smoking enhances the risk for AMD is not known. We speculate that reactive oxygen and nitrogen species derived from tobacco smoke in the lungs leads to oxidative protein modifications in the blood that contribute to drusen formation and choroidal neovascularization. Results from a recent clinical trial (5) also demonstrate that the progression of AMD can be slowed in some individuals by high daily doses of antioxidant vitamins and zinc. Direct evidence of oxidative damage in AMD donor eye tissues include elevated levels of CEP adducts uniquely derived from the oxidative fragmentation of DHA (6).

As an approach to better understanding the role of DHA-derived oxidative damage in AMD, we have prepared CEP-modified proteins and anti-CEP antibodies and initiated com-
Comparative analysis of CEP immunoreactivity and autoantibody titer in human plasma. Paal-Knorr condensation of DOHA with the H9280-amino group of lysyl residues was used to prepare CEP-adducted peptides and proteins. The unambiguous synthesis of DOHA and its phosphatidylcholine ester (DOHA-PC) was confirmed by NMR as was the unambiguous production of CEP and CEP-PC-modified dipeptide (Supplemental Material). DOHA was then used to chemically modify KLH, BSA, HSA, and GPDH to yield CEP-adducted antigen and/or reference proteins containing from 1 to 1.6 mol of pyrrole per mol of protein. To confirm further the presence of CEP adducts in DOHA-modified HSA, the modified protein was digested with trypsin, and CEP adducts were localized to HSA residues Lys183 and Lys236 by MALDI-TOF and tandem electrospray mass spectrometric analyses.

CEP Antibody Selectivity—The utility of anti-CEP antibodies for the detection of DHA-derived CEP adducts in biological samples depends on their ability to discriminate between CEP and similar 2-(o-carboxyalkyl)pyrrole modifications generated from other lipid oxidation products such as CPP and CHP adducts (Fig. 1). We found that our polyclonal anti-CEP and anti-CPP antibodies recognize their respective epitopes with excellent discrimination despite the fact that they differ by only a single CH2 group. This remarkable structural selectivity persisted when another protein (i.e. CEP-GPDH) was used to coat ELISA wells instead of CEP-BSA. To provide the observed discrimination between these very similar epitopes, the fit must be tight and include strong, geometrically rigorous interactions of the carboxyl groups in the CEP and CPP structures. In view of this exquisite selectivity, it is not surprising that both antibodies cross-react very weakly (0.05–0.24%) with CHP-HSA that contains a much larger carboxyalkyl group than either CEP or CPP. Neither antibody cross-reacts with PP-ACA-HSA that lacks a carboxyl (Table I). The utility of the anti-CEP antibody in Western blot analyses was previously demonstrated at an antibody concentration of 1 g/ml (6).

Immunocytochemical Localization of CEP in Retina—DHA is not uniformly distributed throughout the retina but, rather, is concentrated in the photoreceptor rod outer segments and
the RPE (25, 26). Notably this DHA-rich region of the retina is exposed to high levels of oxygen and is responsible for capturing photons (i.e. phototransduction). Immunocytochemistry demonstrated that these cell layers exhibited intense CEP immunoreactivity in mouse retina (Fig. 5). Normal human retina also exhibited CEP immunoreactivity in photoreceptor cells, but the intensity was much greater in AMD retina. Each RPE cell interacts with the tips of about 50 photoreceptor outer segments, and 10% of each outer segment is shed daily and phagocytized by the RPE. This process is thought to be in part a repair mechanism for the high photooxidative stress prevailing in the retina and certainly introduces significant amounts of oxidatively modified proteins and DHA-containing lipids from the photoreceptor cells into the RPE. The RPE forms an integral part of the blood-retinal barrier and is responsible for vectorial transport of nutrients to the photoreceptor cells and waste products to the blood. We hypothesize that normal protective mechanisms against oxidative stress are compromised in AMD, resulting in oxidative damage that impairs the ability of the RPE to process and degrade waste products from the photoreceptors. A consequence of this impairment appears to be increased deposition of CEP-modified proteins in the adjacent Bruch’s membrane (6) and circulatory system (Fig. 6).

**CEP Adducts Are Generated in Low Yield—**After 24–72 h in vitro oxidation of DHA or DHA-PC with HSA, we found the yield of CEP adduct to be low, namely in the range of −0.7–4 ppm relative to the DHA or lipid and −8–50 ppm relative to HSA. Saponification was required for detection of the higher levels from DHA-PC. These very low levels of CEP modifications are due in part to the complicated array of DHA oxidation products that compete with formation of the HOHA and HOHA-PC fragmentation products that produce CEP adducts (12). Reactions with other DHA oxidation products can result in alternative protein modifications such as Michael adducts, Schiff bases, or protein cross-links (6, 12). In vitro reaction of HOHA-PC with HSA for 24 h yielded significantly greater levels of CEP adducts (−5000 ppm or 0.5% yield relative to the lipid fragment); however, this yield is still relatively low and most likely reflects the susceptibility of HOHA-PC itself to further oxidation and rearrangements (12). In contrast, the yield of CH$_3$HSA adducts (−200 ppm) from linoleate (and HODA) is much higher than CEP. This is due in part to fewer double bonds in linoleate than DHA and consequently fewer possible alternative oxidation products. There is also less possible interference by the carboxyl group with pyrrole generation for HODA due to the greater distance between the incipient pyrrole ring and the carboxyl group compared with HOHA. The absence of carboxyl group interception of intermediates in the pyrrole-forming process may also explain the higher apparent yields of CEP-HSA obtained from oxidation of the phospholipid ester (i.e. DHA-PC) followed by saponification than produced by oxidation of the free acid DHA. These in vitro studies show that CEP adducts are generated from DHA in low yield over a few days. Analyses of human plasma (Figs. 6 and 7) and human ocular tissues (Fig. 5) demonstrate CEP adducts accumulate in vivo and are more abundant in tissues from AMD donors than from normal tissues (6).

**Plasma CEP Immunoreactivity and CEP Autoantibody Titer Correlate with AMD—**We found the mean levels of CEP immunoreactivity and autoantibody titer in AMD plasma statistically to be significantly higher than in controls. In addition, the variability of CEP immunoreactivity and autoantibody was about 2–3 times higher in plasma from AMD donors than from the older controls. This higher variability likely reflects the complex genetic basis of AMD susceptibility and possible environmental differences among the donors. Interestingly, the variability of CEP immunoreactivity was about 30% greater in the plasma from younger controls than from older normal donors. The basis of this higher apparent variability is presently not known. However, AMD is a progressive disease, and we speculate that oxidative modifications such as CEP adducts will gradually increase in the plasma of those susceptible to developing the disease and be detectable before the manifestation of retinal pathology. Our analyses of human plasma show that a large fraction of the AMD donors exhibited both elevated CEP immunoreactivity and high anti-CEP autoantibody titer (Fig. 8). Specifically, 12 of the 19 AMD donors (63%) exhibited both high immunoreactivity and high autoantibody titer compared with only 1 of the 19 age-matched controls (5%). However, high CEP immunoreactivity was not always associated with high anti-CEP autoantibody titer. Select plasma from both AMD or control donors exhibited higher levels of either CEP immunoreactivity or autoantibody titer but not both. Nevertheless, 92% of the individuals exhibiting both antigen and autoantibody levels above the mean for non-AMD controls had AMD (region IV in Fig. 8). Logistic regression also revealed larger probabilities of AMD associated with increased levels of CEP immunoreactivity and autoantibodies. The logistic regression model obtained using the available data as predictors of AMD fit the equation: In(probability of AMD/(1 − probability of AMD)) = −4.80 + 0.26(CEP immunoreactivity) + 0.82(CEP autoantibody titer). The c statistic based on this preliminary model was 0.80 (95% confidence interval), indicating that by using both variables there is an 80% likelihood that a randomly selected person with AMD is likely to receive a higher predicted probability of having AMD than is a person without AMD. This probability was greater than using either CEP immunoreactivity (0.77) or CEP autoantibody titer (0.73) alone. Given the apparently low incidence of false positives, a combination of these two parameters, namely plasma CEP immunoreactivity and autoantibody titer, may have diagnostic utility. A much larger clinical investigation is now warranted to test whether this approach could be useful in predicting AMD susceptibility.

Finally, the presence of elevated levels of CEP autoantibodies in AMD plasma supports suggestions by others that immune-mediated events may be associated with the pathogenesis of AMD (27–29). Over time, immune mediated events may...
be contributory, for example, to the formation of AMD drusen; however, it is not yet clear that such events are causative. We hypothesize that protein modifications derived from the oxidation of lipids and carbohydrates may be primary catalysts in AMD pathology and likely play significant roles in both drusen biogenesis and choroidal neovascularization (6). Immune responses triggered by oxidative lipid-derived protein modification may play a more dominant role in autoimmune pathologies than generally recognized. Plasma analyses for oxidative protein modifications such as CEP and the associated autoantibodies may provide an early warning system for predicting those at risk of developing AMD and facilitate timely therapeutic intervention preventing retinal degeneration and vision loss.

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