Isolevuglandins and Mitochondrial Enzymes in the Retina: Mass Spectrometry Detection of Post-Translational Modification of Sterol-Metabolizing CYP27A1

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We report the first peptide mapping and sequencing of an in vivo isolevuglandin-modified protein. Mitochondrial cytochrome P450 27A1 (CYP27A1) is a ubiquitous multifunctional sterol C27-hydroxylase that eliminates cholesterol and likely 7-ketocholesterol from the retina and many other tissues. We investigated the post-translational modification of this protein with isolevuglandins, arachidonate oxidation products. Treatment of purified recombinant CYP27A1 with authentic iso[4]levuglandin E$_2$ (iso[4]LGE$_2$) in vitro diminished enzyme activity in a time and phospholipid-dependent manner. A multiple reaction monitoring (MRM) protocol was then developed to identify the sites and extent of iso[4]LGE$_2$ adduction. CYP27A1 exhibited only three Lys residues, Lys-134, Lys-358, and Lys-476, that readily interact with iso[4]LGE$_2$ in vitro. Such selective modification enabled the generation of an internal standard, $^{15}$N-labeled CYP27A1 modified with iso[4]LGE$_2$, for the subsequent analysis of a human retinal sample. Two MRM transitions arising from the peptide AVLK$_{358}$(-C$_{20}$H$_{30}$O$_3$)ETLR in the retinal sample were observed which co-eluted with the corresponding two $^{15}$N transitions from the supplemented standard. These data demonstrate that modified CYP27A1 is present in the retina. We suggest that such protein modification impairs sterol elimination and likely has other pathological sequelae. We also propose that the post-translational modifications identified in CYP27A1 exemplify a general mechanism whereby oxidative stress and inflammation deleteriously affect protein function, contributing, for example to cholesterol-rich lesions associated with age-related macular degeneration and cardiovascular disease. The proteomic protocols developed in the present investigation are generally applicable to characterization of lipid-derived oxidative protein modifications occurring in vivo, including proteins bound to membranes.

The mitochondrial enzyme cytochrome P450 27A1 (CYP27A1) is expressed in many tissues where it catalyzes the C27-hydroxylation of cholesterol and other sterols, thereby playing an important role in the maintenance of cholesterol homeostasis, bile acid biosynthesis, and activation of vitamin D$_3$ (reviewed in (1)). In specific organs, the functions of CYP27A1 could also include elimination of cytotoxic 7-ketocholesterol (2,4) and regulation of cholesterol homeostasis (5). Deficiency in CYP27A1 leads to cerebrotendinous xanthomatosis, a lipid storage disease with multiple manifestations including premature atherosclerosis and retinal abnormalities (6-8). Recently, CYP27A1 was found to be expressed in the retina (9,10) and was shown to be the major contributor to enzymatic degradation of cholesterol in this organ (11). Quantification of...
CYP27A1 in human retina revealed that one of its peptides, VVLAPETGELK, was consistently underrepresented by as much as 50% compared to other CYP27A1 peptides (10). However, this peptide underrepresentation was not observed in the human brain (10). We surmised the difference resulted from retina-specific post-translational modification due to the highly oxidative retinal environment and constant exposure to light (12-14).

The retina is known to contain high amounts of polyunsaturated fatty acids (PUFAs), of which arachidonic acid is one of the most abundant (13). Arachidonyl phospholipids (PLs) have been shown to undergo free radical-induced oxidation in vivo to generate isolevuglandins (isoLGs) as demonstrated by the presence of isoLG-protein adducts in human serum (15). Most isoLG isomers such as iso[4]levuglandin E₂ (iso[4]LGE₂) are only generated via free-radical mediated oxidation (16). One particular isomer, levuglandin E₂, may also form through rearrangement of prostaglandin H₂, a product of cyclooxygenase-induced oxidation (17). Regardless of origin, isoLGs represent a family of γ-keto aldehydes (16-18), which are highly reactive towards free primary amines such as the ε-amine of lysine residues in proteins. They form covalent pyrrole-derived adducts with greater avidity than most other lipid oxidation products such as 4-hydroxynonenal (19-21). In the human eye, isoLG-adducted calpain-1 has been shown to accumulate in the trabecular meshwork with this modification abolishing enzyme activity (22).

Existing studies to quantify isoLG post-translational modification in vivo have focused on measuring total lysine-isoLG adducts following complete proteolytic digestion to free amino acids (23). This approach is a useful diagnostic tool for measuring long-term markers of oxidative stress (18). However, it does not indicate which proteins are affected or the lysine residues modified. Furthermore, MALDI-TOF mass spectra of tryptic digests of adducted proteins have only been reported for modification in vitro (24-27). Identification of proteins modified in vivo has only been based on Western blot analysis of two-dimensional SDS-PAGE using antibodies against adducts and subsequent proteomic analysis (28). While the proteins identified co-migrate with immunoreactivity against specific adducts, proteomic evidence establishing the in vivo presence of isoLG-derived modifications of specific aminoacyl residues of proteins in biological samples has never been reported.

Site-specific post-translational protein modifications can be detected and quantified using mass spectrometry-based multiple reaction monitoring (MRM) assay (29). MRM focuses only on specific m/z, while all other m/z are excluded. This allows selection of the appropriate precursor/product ion pairs, or transitions, for the analyte of interest thereby enhancing sensitivity and dynamic range. This methodology has proved particularly successful for profiling phosphorylation (30), acetylation (31), glycosylation (32), and ubiquitination (33) of soluble proteins. Quantification of other types of modification using this approach is rare (27), and do not extend to membrane proteins.

In the present work, we demonstrate how a combination of in vitro studies utilizing purified enzyme of interest and authentic modifying reagent followed by MRM analysis of a biological sample with the 15N-labeled form of the enzyme as an internal standard enables detection of unconventional protein modification. This work creates a paradigm for similar studies on other proteins. Moreover, it provides novel mechanistic insight into how oxidative stress/inflammation could impair cholesterol elimination from the retina and lead to formation of cholesterol-rich drusen, a hallmark of age-related macular degeneration (AMD) (34,35).

EXPERIMENTAL PROCEDURES

Materials - Ammonium chloride (15N, 99%) was purchased from Cambridge Isotope Laboratories (Andover, MA). DC Protein Assay kit was from Bio-Rad Laboratories (Hercules, CA). Sequencing grade trypsin and chymotrypsin were from Promega Corp. (Madison, WI). Complete, EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN). 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Authentic iso[4]LGE₂ was synthesized as
described and assayed by NMR (16). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Post-mortem human eyes were obtained from the Georgia Eye Bank followed by isolation of retina as described (11) within 16 h from time of death. Human tissue use conformed to the Declaration of Helsinki and institutional review at Case Western Reserve University. The donor (PM28) was an 86 year-old, Caucasian male diagnosed with dry AMD in both eyes. Whole bovine eyes were obtained from local slaughterhouses, and the retinas were isolated as described (11).

Expression and Purification of CYP27A1 - Natural isotopic abundance (NIA) and \(^{13}\)N-labeled recombinant human CYP27A1 were expressed as described (10,36). The P450 concentrations were calculated from the CO-reduced difference spectra using an absorption coefficient of 91 mM\(^{-1}\) cm\(^{-1}\) between \(A_{450}\) and \(A_{490}\) (37).

Treatment of CYP27A1 with Iso[4]LGE\(_2\) in the Absence of PLs and Subsequent Characterization - In all descriptions of treatment of CYP27A1 with iso[4]LGE\(_2\), molar excesses are expressed relative to protein lysine content (23 mol lysine/mol of CYP27A1). Purified recombinant NIA-CYP27A1 (5 nmol) was added to 50 mM potassium phosphate buffer (KP\(_i\), pH 7.2, containing 100 \(\mu\)M diethylene triamine pentaacetic acid (DTPA) and a 10-fold excess of iso[4]LGE\(_2\) (1.150 \(\mu\)mol) in a final volume of 1 mL. The reaction mixture was kept at room temperature with gentle shaking for 120 min then quenched with 100 mM glycine to neutralize unreacted iso[4]LGE\(_2\). A portion of the mixture was used for measuring the reduced CO difference spectrum and assay of enzyme activity. The remainder was flash frozen in liquid nitrogen and stored at -80 °C for further analysis by MS. The control incubation lacked iso[4]LGE\(_2\). Enzyme activity was assessed in the in vitro reconstituted system consisting of 200 pmol CYP27A1, 6,000 pmol adrenodoxin, 800 pmol adrenodoxin reductase, 11 pmol [\(^3\)H]cholesterol and 38 \(\mu\)g/mL DLPC. The conversion of cholesterol to 27-hydroxycholesterol was measured by HPLC with inline \(\beta\) detection as described previously (38).

For LC-MS/MS analysis, in-gel and in-solution digests were performed using either trypsin or chymotrypsin. For in-gel digest, NIA-CYP27A1 (15 pmol) was subjected to 10% SDS-PAGE. The gel was stained with Coomassie Blue and a region corresponding to proteins with molecular weight between 50 to 60 kDa was excised, destained, reduced in 20 mM DTT for 30 min at room temperature, and alkylated in 100 mM iodoacetamide in the dark for 30 min at room temperature. The protein was then digested by chymotrypsin or trypsin for 22 h at 37 °C at a protease to P450 ratio of 1:50 (w/w) in 50 mM NH\(_4\)HCO\(_3\), pH 7.8 buffer. Peptides were extracted with 50% acetonitrile containing 5% formic acid, dried in a vacuum concentrator, and stored at -20 °C. For in-solution digest, NIA-CYP27A1 (1 nmol) was mixed with 1 nmol of unmodified \(^{13}\)N-CYP27A1. The protein solution was dialyzed against 50 mM KP\(_i\), pH 7.2, containing 100 \(\mu\)M DTPA, 0.2 M NaCl, overnight to remove detergent and glycerol. The sample was concentrated to a volume of 5 \(\mu\)L in an Amicon Ultra centrifugal filter device (regenerated cellulose, 50 kDa molecular weight cut-off, Millipore, Billerica, MA) followed by protein denaturation in 8 M urea, reduction in 10 mM DTT for 30 min at room temperature, and alkylation in 25 mM iodoacetamide in the dark for 30 min at room temperature. The protein was then digested by chymotrypsin or trypsin for 22 h at 37 °C at a protease to P450 ratio of 1:50 (w/w) in 10 mM Tris-HCl, pH 8.0, containing 0.8 M urea. The digest solution was applied to Ultra-Micro PrepTips (C\(_{18}\) reverse phase extraction pipette tip columns, The Nest Group, Southborough, MA) to remove residual salt and detergent. Peptides were dried in a vacuum concentrator, and stored at -20 °C. Peptide separations were performed on an Ultimate 3000 LC system with a C\(_{18}\) Acclaim PepMap 100 column (0.075 × 150 mm, Dionex, Sunnyvale, CA). Peptides were eluted over a 50 min gradient from 0% to 80% acetonitrile in water, containing 0.1% formic acid, at a flow rate of 300 nL/min. The column effluent was continuously directed into the nanospray source of the mass spectrometer, a hybrid Fourier transform ion cyclotron resonance (FTICR)/linear ion trap mass spectrometer (LTQ FT Ultra, Thermo Scientific, West Palm Beach, FL). The following parameters were used for all acquisition methods on the LTQ FT Ultra MS: an ion spray voltage of 2400 V and an interface capillary heating
temperature of 200 °C. Full mass spectra were acquired from the FTICR, and the tandem mass spectra (MS/MS) of the 8 most intense ions were recorded by the linear ion trap data dependent mode with normalized collision energy of 35 eV, isolation width of 2.5 Da, and activation Q of 0.25.

Mascot Database Search to Identify Modified Peptides - Peptides were identified from LTQ FT Ultra MS/MS experimental data by generating peak lists with Mascot Daemon and submitting these to the Mascot search engine, version 2.3.0 (Matrix Science, Boston, MA, www.matrixscience.com). S-carbamidomethylation of cysteine was set as a fixed modification while oxidation of methionine (methionine sulfoxide) was set as a variable modification. Formulas corresponding to the various oxidation and dehydration states of iso[4]LGE$_2$ adducts (39) were manually entered into the Mascot residue modification database and selected as variable modifications for lysine (Table S1, structures shown in Figure S1). Mass tolerances were ± 15 ppm for precursor ions and ± 0.8 Da for fragment ions. One missed cleavage site was allowed for trypsin (cleaves at residues Lys and Arg, but not at Pro) and three missed cleavage sites for chymotrypsin (cleaves at Phe, Leu, Trp, and Tyr, but not at Pro). Searches were restricted to a sequence database containing only human CYP27A1 (Swiss-Prot Number Q02318, residues 34-531) since purified protein was used. Only peptides with a significant score greater than 13 (p <0.05) according to Mascot’s scoring algorithm were considered.

Isolation of Mitochondrial PLs - Isolation of mitochondria from bovine retinal tissue was adapted from an established protocol (40). Fresh retina (10g wet tissue) was homogenized with a Teflon pestle on ice in 20 mL of homogenization buffer consisting of 50 mM Tris-Cl, pH 7.4, containing 250 mM sucrose, 5 mM MgCl$_2$, 1 mM PMSF, 1 mM DTT, 1 tablet of the protease inhibitor cocktail per 50 mL, and 100 µg/mL butylated hydroxytoluene. Total mitochondrial lipids were extracted by vortexing mitochondria in 32 mL Folch reagent (chloroform:methanol, 2:1, v/v) for 30 min (41). To aid phase separation, 2 mL of 150 mM NaCl was added to the suspension, vortexed for 5 min, and centrifuged at 1000g for 10 min. The lower organic phase was transferred to a clean glass tube. The aqueous phase was re-extracted 2 more times by vortexing with 16 mL Folch reagent for 30 min, adding 2 mL of 150 mM NaCl and vortexing for 5 min followed by centrifugation at 1000g for 10 min, and transferring the organic phase to a clean glass tube. All organic phases were pooled and dried under vacuum (Savant SC210A SpeedVac Concentrator, Thermo Scientific, Asheville, NC), yielding 0.054 g of lipids. The residue was then dissolved in 500 µL of chloroform and loaded onto a 690 mg silica solid phase extraction cartridge (Sep-Pak® Classic Silica, Waters) for separation (42). Nonphosphorous lipids were washed from the cartridge with 20 mL chloroform. PLs were eluted with 30 mL methanol and concentrated under vacuum, yielding 0.044 g of lipids. PLs were dissolved in methanol (10 mg/mL) and stored at -20 °C. Purity of PLs and nonphosphorous lipids was assessed by silica thin layer chromatography with a mobile phase consisting of hexane:diethyl ether:methanol:acetic acid (90:20:5:2, v/v/v/v) and detected by staining with iodine vapor as described (43).

Reconstitution of CYP27A1 into Liposomes - Stock solutions of retinal mitochondrial PLs, a mixture of DLPC and DOPE (4:3, w/w), and DLPC alone were prepared by dissolving 10 mg of PLs in methanol. PLs (1.25 mg, approx. 1800 nmol) were dispensed into a glass tube and the solvent evaporated under nitrogen. PLs were hydrated for 10 min in 200-500 µL of 50 mM KP$_2$, pH 7.2, containing 100 µM DTPA, briefly vortexed, and sonicated for 30 min at 50% power (Digital Sonifier S-450D, Branson Ultrasonics, Danbury, CT) (44). Purified recombinant CYP27A1 (5 nmol) was added to the liposome
solution and allowed to incorporate for 30 min at 25 °C.

Treatment of CYP27A1 with Iso[4]LGE2 in the Presence of PLs and Subsequent Characterization
- The P450-liposome mixture (5 nmol P450) was incubated with a 2-fold molar excess of iso[4]LGE2 in a final volume of 1 mL at room temperature with gentle shaking. Aliquots were taken at different time points, quenched, and used for analysis by: 1) the CO-reduced difference spectrum, 2) assay of enzyme activity, and 3) MRM. Enzyme activity was measured as described above except no extra DLPC was added. For MRM analyses, modified NIA-CYP27A1 (100 pmol) was mixed with an equimolar amount 15N-CYP27A1 and digested with trypsin over a 42 h gradient from 1% to 31% acetonitrile in water containing 0.1% formic acid at a flow rate of 300 nL/min. The column effluent was continuously directed into the nanospray source of the mass spectrometer, a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP, ABI/MDS-Sciex, Carlsbad, California). All acquisition methods used the following parameters: an ion spray voltage of 2100 V, curtain gas of 30 psi, source gas of 9 psi, interface heating temperature of 170 °C, declustering potential of 76 V for +2 precursor ions and 65 V for +3 precursor ions, collision energy of 30 eV for +2 precursor ions and 22 eV for +3 precursor ions, and collision cell exit potential of 16 V for +2 precursor ions and 13 V for +3 precursor ions. The dwell time for all transitions was 40 ms.

The mass spectrometer monitored three transitions per peptide. Selection of transitions was as described previously (45). The identities of the measured peptides were confirmed based on two parameters of the internal standard which was run under the same conditions: 1) the retention time of the three MRM peaks from a given peptide, and 2) the ratio among the three MRM peaks. The mean and standard deviation were calculated by treating the three transitions for each of the target peptides and the three experimental replicates all as independent measurements.

Preparation of 15N-CYP27A1 Modified with Iso[4]LGE2 - 15N-CYP27A1 (1 nmol) in 50 mM KP, pH 7.2, containing 100 µM DTPA was incubated with an equimolar amount of iso[4]LGE2 in a final volume of 0.2 mL at room temperature with gentle shaking for 15 min. The reaction mixture was quenched by addition of 1 µL of 100 mM glycine. The mixture was flash frozen in liquid nitrogen and stored at -80 °C.

Processing of the Human Retina - Retina was placed in 25 mM NH₄HCO₃ and homogenized by sonication at 30 W using three continuous 10 s cycles (Sonicator 3000, Misonix Inc., Farmingdale, NY). The total protein concentration was measured in the presence of 2% (w/w) SDS using the DC protein Assay kit and bovine serum albumin as a standard. The homogenate was centrifuged at 153 000 g for 30 min, and the resulting total membrane pellet was frozen and stored at -80 °C. For proteolytic hydrolysis, the total membrane pellet was thawed and resuspended in 25 mM NH₄HCO₃, pH 7.9, containing 0.2% (w/w) sodium cholate and supplemented with modified 15N-CYP27A1 (25 pmol) as an internal standard. The samples were heated at 90 °C for 5 min, cooled to room temperature, and treated with trypsin for 15 h at 37 °C. The trypsin to P450 ratio was 1:50 (w/w). After trypsinolysis, the samples were centrifuged at 153 000g for 30 min, and the supernatants were transferred to new tubes. Each supernatant was then treated with 0.5% w/v TFA for 30 min at 37 °C and centrifuged again at 153 000g for 30 min. The supernatants from the second centrifugation were transferred to new tubes again, mixed with an equal volume of acetonitrile, and dried using a Vacufuge (Eppendorf AG, Hamburg, Germany).

RESULTS AND DISCUSSION

Characterization of CYP27A1 After Treatment with Iso[4]LGE2 in the Absence of PLs - The effect of the iso[4]LGE2 treatment (10-fold molar excess for 120 min) on functional properties of CYP27A1 was assessed using two assays: the CO-reduced difference spectrum which reflected the content of the functional P450, and an enzyme assay which...
reflects the ability of CYP27A1 to hydroxylate cholesterol in a reconstituted enzyme system. Only a peak at 420 nm was observed in the CO-spectrum of the treated CYP27A1 indicating enzyme denaturation under the experimental conditions used. Also, no enzyme product, 27-hydroxycholesterol, was detected in the enzyme assay. In contrast, the P450 content and enzyme activity in control incubations were similar to those observed at the initiation of treatment. These data suggest that the iso[4]LGE2 treatment leads to modification of CYP27A1 and is accompanied by enzyme denaturation and loss of activity. To confirm modification, the treated CYP27A1 was trypsinolyzed in-gel and in-solution followed by FTICR MS/MS. Peak lists from the acquired MS/MS spectra were submitted to the Mascot database search engine. Evaluation criteria for identification of the modified peptides were as follows: 1) a significant peptide score as reported by Mascot’s scoring algorithm (46), 2) the existence of y and b series ions upstream and downstream of the modified lysine in the tandem MS spectra, 3) detection under different types of hydrolysis, and 4) the existence of multiple adduct dehydration and oxidation states. Mascot analysis of the spectra from the in-solution tryptic digest (17% sequence coverage, encompassing 26% of the Lys residues) did not identify any modified peptides with a significant score. In contrast, analysis of the in-gel tryptic digest (72% sequence, 56% Lys coverage) identified 2 distinct peptides, AVLK\textsuperscript{35}ETLR and VVLAPETGELK\textsuperscript{476}SVAR, modified at Lys-358 and Lys-476 (Table 1). In both peptides, modified lysine residues were within the peptide and not at the C-terminus, indicating that modification results in missed tryptic cleavage. Remarkably, the latter peptide is an expansion of VVLAPETGELK\textsuperscript{476} which was consistently underrepresented in our previous study assessing by MRM the CYP27A1 content in human donor retinas (10). To increase sequence coverage, modified CYP27A1 was digested with chymotrypsin in-gel (96% sequence, 100% Lys coverage) and in-solution (95% sequence, 100% Lys coverage). Mascot analysis identified only 1 additional peptide with modification at Lys-134, NQRLLK\textsuperscript{134}PAEAALY, for both in-gel and in-solution chymotrypsin digests, with the later having a significant score (Table 1). Thus, based on our evaluation criteria, Mascot identified only 3 lysine residues out of 23 in CYP27A1 as modified by iso[4]LGE2. Lys-358 was shown previously to be important for interaction with adrenodoxin, the CYP27A1 redox partner (47). Hence modification by iso[4]LGE2 could disrupt this interaction through both steric hindrance and elimination of important electrostatic charges. The functional role of the second lysine residue modified, Lys-476, is likely different. According to the published computational model of CYP27A1 (PDB Number 1MFX) (48), this residue lies along the substrate access channel. Iso[4]LGE2 adducts on Lys-476 may obstruct this channel and prevent cholesterol from reaching the active site. The role of the third lysine residue modified, Lys-134, is not yet clear, but experiments with PLs (next section) indicate that it may be embedded in the mitochondrial membrane.

Characterization of Iso[4]LGE2 Treatment of CYP27A1 Reconstituted into Different PLs - To model membrane insertion, purified recombinant CYP27A1 was reconstituted into liposomes of various composition: bovine retinal mitochondrial PLs, a D LPC and DOPE mixture (4:3, w/w) which approximates the ratio of phosphatidylcholine to phosphatidylethanolamine found in human hepatic mitochondria (49), or DLPC which contains no free amines and is a standard model PL. In this set of experiments, the ratio of iso[4]LGE2 was lowered to a 2-fold molar excess, and kinetics investigated. Enzyme activity was decreased sharply by 75% at 5 min. Yet the P450 content decreased only by 25%, indicating that modification impaired activity without completely denaturing the protein. Both enzyme activity and P450 content plateaued after 15 min with little further reduction (Fig. 1). This is in contrast to the treatment with a 10-fold molar excess of iso[4]LGE2 leading to complete P450 denaturation and loss of enzyme activity. Such effects of high iso[4]LGE2 concentrations could be attributable to modification of additional, less reactive lysine residues resulting in peptides with multiple missed cleavage sites. Such peptides are difficult to unambiguously identify with Mascot because the number of false positive hits increases as three or more missed cleavage sites are allowed. Furthermore, at high iso[4]LGE2 concentrations some arginine and histidine residues could be
modified. As the chemical structure of these modifications has not been demonstrated, Mascot search criteria could not be created. Consequently, these putative modifications were not investigated.

At each time interval, aliquots were withdrawn and spiked with the internal standard, \( ^{15}\text{N}-\text{CYP27A1} \), in an amount approximately equimolar to NIA-CYP27A1. Proteins were subjected to proteolyses followed by quantitative assessment by MRM. The ratios of transition intensities between the NIA peptides and corresponding \( ^{15}\text{N} \) peptides were calculated and assumed to be equal to the proteins’ initial mixing ratio for NIA peptides not possessing modification and decreased for modified peptides proportionally to the extent of modification. Peptides that did not contain lysine residues and therefore could not be modified by iso[4]LGE were analyzed first (Group I, Fig. 2). Within an aliquot (Z axis in Fig. 2), the ratio between these peptides varied up to 17% due to MRM quantification error (Table S2). The ratios also varied up to 19% from aliquot to aliquot (X axis in Fig. 2) likely due to pipeting errors. Because ratios were calculated independently for each time point, pipeting uncertainties did not contribute to uncertainties in quantification. Mixing ratios in Group I were then used for calculating lysine residue modification in other peptides which were grouped based on sequence and observed changes in mixing ratio. Group II consisted of only one peptide, VVLAPETGELK\(^{476} \), in which the ratio for the 5 min reaction time was 0.69 while that in Group I was 0.84 indicating that 18% of the peptide was modified (Fig. 2, Table S2). The extent of modification of this peptide further increased to 34% and 40% after 15 min and 120 min, respectively. Thus changes in both enzyme activity and peptide modification level off after 15 min of iso[4]LGE\(^2 \) treatment. Group III is comprised of three lysine-containing peptides (WTRPVLPFWK\(^{236} \), YLDGWNAIFSFGK\(^{250} \), and EIEVGDGLFPK\(^{387} \) that showed either no modification or modification up to 16% (which is within experimental variation). This was substantially less than in Group II. Finally, peptides that contain both lysine and methionine residues formed Group IV because of possible background oxidation of the latter. SIGLMFQNSLYATFLPK\(^{226} \) did not appear to be either modified or oxidized as indicated by no decrease in the ratio compared to Group I. Conversely, NDMELWK\(^{101} \) and LLK\(^{134} \)PAEAALYTDAFNEVIDDFMTR seemed to have methionine oxidation since the ratios in the control aliquot were lower than in Group I. DFAHMPLLK\(^{354} \) was likely unmodified in the presence of retinal mitochondrial PLs as the mixing ratio was close to experimental error. Furthermore, the presence of methionine in the peptide introduced additional uncertainty in estimating the extent of modification.

This analysis was repeated for CYP27A1 reconstituted into liposomes composed of a mixture of DLPC and DOPE or DLPC alone (Figs 3, 4, Tables S3, S4). In all three types of PLs, Lys-476 was the most extensively modified as assessed by quantitative disappearance of the peptide VVLAPETGELK\(^{476} \). This is in agreement with direct observation of the modified peptide VVLAPETGELK\(^{476}(-\text{C}_2\text{H}_5\text{O}_3\text{S})\text{SVAR} \) in the FTICR MS experiments. Moreover, Group II and Group III peptides showed higher degrees of modification when CYP27A1 was incorporated into DLPC. Most Group III peptides showed no modification in mitochondrial PLs and the DLPC-DOPE mixture, which both contained primary amines. This suggests that the modification is PL dependent, likely due to a combination of differing CYP27A1 membrane insertion modes and utilization of iso[4]LGE\(^2 \) for both protein and PL modification.

To generate Lys-134 and Lys-354-encompassing peptides which do not contain methionine, and a Lys-358 peptide, which was unobservable under tryptic hydrolysis, we mixed NIA-CYP27A1 modified in the presence of mitochondrial PLs with an equimolar amount of \( ^{15}\text{N}-\text{CYP27A1} \) and performed chymotryptic hydrolysis. The average mixing ratio was calculated using two peptides: LRNSQPATPRIQHPF\(^{431} \) and LFPK\(^{387} \)NTQF (Fig. 5, Table S5). Three peptides, LK\(^{134} \)PAEAALY, K\(^{134} \)PAEAALY, and NQRLLK\(^{134} \)PAEAALY, were monitored and showed unchanged ratios indicating that Lys-134 was not modified under these conditions. Lack of modification at Lys\(^{134} \) may be due to the lower molar excess of isoLG and residue inaccessibility after insertion into the liposome. Ratios for LK\(^{354} \)AVLK\(^{358} \)ETL were lower by at least 30%
(Table S5) in comparison to average mixing ratio, suggesting modification. Modification is likely assignable to Lys-358 due to direct observation of adducted Lys-358 in the FTICR MS experiment and the small change in mixing ratios for the Lys-354 peptide in the presence of retinal mitochondrial PLs in the trypic hydrolysis experiments. K$^{+}$SVARILV ratios were also lower by at least 20%, in agreement with the results of the trypic hydrolysis.

Overall, the data demonstrate that PL-reconstituted CYP27A1 possesses two lysine residues, Lys-358 and Lys-476, that interact even with a 2-fold molar excess of iso[4]LGE$_2$. Modification leads to missed cleavage and an increased $m/z$, thus lowering the yield of Lys-358 and Lys-476 containing peptides. These studies justified our subsequent experiments aimed at identification of iso[4]LGE$_2$ modification in the retina.

**Preparation and Characterization of the Iso[4]LGE$_2$ Modified $^{15}$N-CYP27A1 Internal Standard** - To unambiguously detect iso[4]LGE$_2$-CYP27A1 adducts in a biological sample, a $^{15}$N-labeled internal standard with modified Lys$^{358}$ and Lys$^{476}$ should be used. This standard should have minimal modification at flanking lysine residues which could lead to complex peptide patterns through multiple missed cleavage sites resulting from adduction. Therefore, we further lowered the excess of iso[4]LGE$_2$ over protein lysine content to a 1:1 molar ratio. Modified $^{15}$N-CYP27A1 was subjected to trypic hydrolysis followed by MRM monitoring of 61 transitions arising from AVLK$^{358}$-(C$_{20}$H$_{39}$O$_{3}$)ETLR and VVLAPETGELK$^{358}$-(C$_{20}$H$_{39}$O$_{3}$)SVAR (Table S6). The list of transitions was generated by the R software package OrgMassSpecR ([http://orgmassspec.r-forge.r-project.org](http://orgmassspec.r-forge.r-project.org)). We indeed observed multiple transitions for both peptides adducted with iso[4]LGE$_2$ (data not shown) indicating modification still occurred with an even lower amount of iso[4]LGE$_2$.

Modified $^{15}$N-CYP27A1 was then mixed with an equimolramount of NIA-CYP27A1 treated with a 2-fold molar excess of iso[4]LGE$_2$ for different times in the presence of mitochondrial PLs. The mixtures were hydrolyzed with trypsin and monitored by MRM. The four most intense transitions for the NIA and $^{15}$N-CYP27A1 peptides AVLK$^{358}$-(C$_{20}$H$_{39}$O$_{3}$)ETLR and VVLAPETGELK$^{358}$-(C$_{20}$H$_{39}$O$_{3}$)SVAR were identified. For each peptide, the transition with lowest intensity was assigned a value of 1.0 (shown in bold in Table 2) and the intensities of the three other transitions were scaled proportionally. The scaled intensities for NIA-AVLK$^{358}$-(C$_{20}$H$_{39}$O$_{3}$)ETLR and VVLAPETGELK$^{358}$-(C$_{20}$H$_{39}$O$_{3}$)SVAR were very similar to the intensities from the corresponding $^{15}$N peptide at all treatment times (summarized as average in Table 2). This analysis indicated that the MRM characteristics of modified $^{15}$N-CYP27A1 were similar to that of modified NIA-CYP27A1 and that the former could be used for detection of this adduct in biological samples.

**Detection of Iso[4]LGE$_2$-CYP27A1 Adducts in Human Retina** - Initially, the total membrane pellet from a portion of the human neural retina was supplemented with modified $^{15}$N-CYP27A1 and treated with trypsin. Unfortunately, MRM analysis revealed transitions only from the internal standard indicating a need for sample enrichment compatible with MRM workflow. Therefore, the total membrane pellet was separated by preparative 10% SDS-PAGE, and a Mini Whole Gel Eluter (Bio-Rad, Hercules, CA) was used to collect 14 fractions following the manufacturer's protocol. The fractions were re-analyzed by 10% SDS-PAGE to determine the molecular weight range of each (Fig. S2). Fractions #7 and #8, encompassing approximately 50 kDa to 65 kDa proteins, were pooled for further analysis based on the molecular mass of CYP27A1 (57 kDa). This pooled fraction was precipitated by chloroform/methanol, treated with trypsin, and subjected to MRM. A list of transitions monitored is summarized in Table S7. MRM detected two transitions from the AVLK$^{358}$-(C$_{20}$H$_{39}$O$_{3}$)ETLR peptide in the retinal sample and the corresponding two transitions from the iso[4]LGE$_2$ modified $^{15}$N-AVLK$^{358}$-(C$_{20}$H$_{39}$O$_{3}$)ETLR peptide from the internal standard (Fig. 6). All the transitions were eluted at the same retention time. The observed y$_{6+4}$ and y$_{4+1}$ product ions encompassed regions of the precursor ion upstream and downstream of the modification, respectively. This is the first direct proof of isoLG modification in human retina and the first peptide
mapping and sequencing of an in vivo isoLG-modified protein.

The \((C_{30}H_{56}O_3)\)-isobaric adduct that we detected in human retina could arise either from modification by iso[4]LGE2, which is generated non-enzymatically via free-radical oxidation of arachidonyl phospholipids (21), or from modification with the isoLG isomer LGE2, which could also be generated by the cyclooxygenase pathway (reviewed in (18). The latter reflects an inflammatory process and may certainly have occurred in the retinal sample of our 86 year old donor afflicted by age-related macular degeneration, a disease whose pathogenesis involves chronic inflammation (50). While identification of the specific isoLG isomers involved in modification of CYP27A1 in the retina is underway in this laboratory, the deleterious effects of the isoLG adduction on enzyme function is consistent with retinal abnormalities developed in individuals with CYP27A1 deficiency (51). Furthermore, owing to age-related systemically elevated levels of oxidative injury, isoLGs could also modify CYP27A1 in vascular endothelium and macrophages, where it is abundant and plays an anti-atherogenic role by eliminating cholesterol (52,53) and the autooxidation product, 7-ketocholesterol (2,3), which is implicated in initiation of apoptosis and inflammation. IsoLG modifications in vascular endothelium and macrophages would explain premature atherosclerosis observed in individuals lacking CYP27A1 (51).

IsoLGs promiscuously modify protein amino groups. Therefore, it is likely that isoLG-modification affects not only CYP27A1 but other proteins including those involved in maintenance of cholesterol homeostasis. Such modification could interfere with protein function, as suggested by the present work and/or trigger other events as suggested by previous studies. IsoLG modification was shown to increase resistance to proteosomal degradation (22) and elicit immunogenic (24) and cytotoxic (54) responses to isoLG adducted molecules, thus leading to impaired cellular health. We propose that increased oxidation of PUFAs represents a common link between aging/oxidative stress/inflammation and AMD as well as cardiovascular disease (CVD) (Fig. 7). Consequently, availability of methodologies that enable identification of individual isoLG-modified proteins and sites of modification on the protein molecule will significantly facilitate investigation of this link and enhance our understanding of the mechanisms underlying wide-spread diseases such as AMD and atherosclerosis.

In summary, in the present work we investigated whether CYP27A1, mediating cholesterol removal from extrahepatic tissues, is modified by oxidized arachidonic acid in vitro and in the human retina. We first established that iso[4]LGE2 treatment of purified recombinant CYP27A1 in solution abolished enzyme activity and led to the formation of iso[4]LGE2 adducts. Next, we reconstituted CYP27A1 in different PLs and ascertained that the enzyme is also modified even when other biological amines were present and iso[4]LGE2 concentrations were lower. MRM analysis revealed that modification in CYP27A1 occurred mostly at three residues, Lys-134, Lys-358, and Lys-476, of which Lys-358 is known to interact with the redox partner and is important for enzyme activity (47). Then, \(^{15}N\)-CYP27A1 was generated, treated with iso[4]LGE2, and confirmed to be a suitable internal standard. Finally, a sample of AMD-afflicted human retina was supplemented with iso[4]LGE2-modified \(^{15}N\)-CYP27A1, enriched and shown by MRM to contain isoLG-modified NIA-CYP27A1. This finding enhances our understanding of the significance of PUFA oxidation, provides novel insight into mechanisms contributing to AMD and CVD, and creates the basis for our future studies investigating whether CYP27A1-isoLG levels could serve as marker for both oxidative stress and impaired cholesterol elimination. The analytical protocol developed in the present work is applicable to investigation of other proteins and other types of modification and is expected to have broad application.

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REFERENCES


**FOOTNOTES**

2 This work was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Case Western Reserve University School of Medicine.
4 Present address: Expression Pathology Inc., Rockville, MD 20850, USA
8 This work was supported in part by grants from the National Institutes of Health (EY018383 and AG024336 to I.A.P, and GM21249 to R.G.S.), an NIH Training Grant fellowship (T32 EY007157 to
C.D.C), and the Case Visual Sciences Research Center NIH Core Grant (P30 EY11373). I.A.P is a recipient of the Jules and Doris Stein Professorship from the Research to Prevent Blindness Foundation.

Certain commercial materials, instruments, and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology (NIST) nor does it imply that the materials, instruments, or equipment identified is necessarily the best available for the purpose.

The abbreviations used are: AMD, age-related macular degeneration; CYP27A1, cytochrome P450 27A1; CVD, cardiovascular disease; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DTPA, diethylene triamine pentaacetic acid; Iso[4]LGE₂, iso[4]levuglandin E₂; IsoLGs, isolevuglandins; KP, potassium phosphate buffer; FTICR, Fourier transform ion cyclotron resonance mass spectrometry; MRM, multiple reaction monitoring; NIA, natural isotopic abundance; PLs, phospholipids; PUFAs, polyunsaturated fatty acids.
FIGURE LEGENDS

Figure 1. Effect of iso[4]LGE2 treatment (2-fold molar excess) on P450 content (dashed line) and enzyme activity (solid line) reconstituted into retinal mitochondrial PLs. Values are expressed as a percentage relative to control (CYP27A1 incubated without iso[4]LGE2). Enzyme activities are the average of 3 experiments ± SEM.

Figure 2. Effect of iso[4]LGE2 treatment and PL Content (Retinal Mitochondrial PLs) on the $^{15}$N:NIA-CYP27A1 ratios as assessed by MRM. Each tryptic peptide is represented by a separate color across all time points. Peptides from Group I are represented in shades of blue, and the Group II peptide is shown in orange. Group III peptides are depicted in shades of green, and Group IV peptides are shown in shades of brown. Data labels have been rounded to one digit after the decimal and some labels have been omitted for clarity. For all data points and statistical treatment, see Table S2.

Figure 3. Effect of iso[4]LGE2 treatment and PL Content (DLPC-DOPE mixture) on the $^{15}$N:NIA-CYP27A1 ratios as assessed by MRM. Each tryptic peptide is represented by a separate color across all time points. The color code for the peptides is the same as in Fig. 2. Data labels have been rounded to one digit after the decimal and some labels have been omitted for clarity. For all data points and statistical treatment, see Table S3.

Figure 4. Effect of iso[4]LGE2 treatment and PL Content (DLPC) on the $^{15}$N:NIA-CYP27A1 ratios as assessed by MRM. Each tryptic peptide is represented by a separate color across all time points. The color code for the peptides is the same as in Fig. 2. Data labels have been rounded to one digit after the decimal and some labels have been omitted for clarity. For all data points and statistical treatment, see Table S4.

Figure 5. Effect of iso[4]LGE2 treatment and PL Content (Retinal Mitochondrial PLs) on the $^{15}$N:NIA-CYP27A1 ratios with chymotryptic hydrolysis. Each peptide is represented by a separate color across all time points. Non-modified peptides are shown in shades of gray, and Lys-134 containing peptides are shown in shades of yellow. The Lys-358 and Lys-476 peptides are shown in peach and orange, respectively. Data labels have been rounded to one digit after the decimal and some labels have been omitted for clarity. For all data points and statistical treatment, see Table S5.

Figure 6. Extracted ion chromatograms from human retina supplemented with modified $^{15}$N-CYP27A1. Transitions from [NIA] and [$^{15}$N] AVLK(-C$_{20}$H$_{36}$O$_{3}$)ETLR were simultaneously monitored (shown in blue and red, respectively). Transitions arising from endogenous isoLG-adducts were observed at identical retention times as those arising from the iso[4]LGE$_{2}$-modified $^{15}$N-CYP27A1 internal standard.

Figure 7. Putative Cascade of Events Initiated by Increased Production of Oxidized PUFAs.
Table 1. Peptides of CYP27A1 Modified by a 10-Fold Molar Excess of Iso[4]LGE₂ as Identified by Mascot Analysis

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Adduct</th>
<th>m/z obs (Charge)</th>
<th>Mr expect</th>
<th>ppm</th>
<th>Score</th>
<th>Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVL̵15̷ETLR</td>
<td>C₂₀H₂₅O₃</td>
<td>622.3873 (+2)</td>
<td>1242.7600</td>
<td>1.05</td>
<td>33</td>
<td>trypsin, in-gel</td>
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<tr>
<td>VVLAPETGEL̵47̷SVAR</td>
<td>C₂₀H₂₅O₃</td>
<td>628.3683 (+3)</td>
<td>1882.0831</td>
<td>0.85</td>
<td>39</td>
<td>trypsin, in-gel</td>
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<tr>
<td>NQRLL̵13̷PAEAALY</td>
<td>C₂₀H₂₈O₅</td>
<td>918.0230 (+2)</td>
<td>1834.0318</td>
<td>4.07</td>
<td>38</td>
<td>chymotrypsin, in-solution</td>
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</tbody>
</table>

Table 2. Scaled Transition Intensity of Two Tryptic Peptides from an Equimolar Mixture of Modified NIA-CYP27A1 and Modified $^{15}$N-CYP27A1. Lowest intensity transitions shown in bold.

<table>
<thead>
<tr>
<th>Transition</th>
<th>Scaled Transition Intensity (Q1:Q3/Q1;Qlowest)</th>
<th>NIA-CYP27A1 Treatment Time</th>
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<tbody>
<tr>
<td></td>
<td>Q1 m/z</td>
<td>Q3 m/z</td>
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<tr>
<td>AVLK$^{15}$N(-C$<em>{20}$H$</em>{26}$O$_{3}$)ETLR (+2)</td>
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<tr>
<td>NIA</td>
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<td></td>
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<td>541.32</td>
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<tr>
<td>VVLAPETGELK$^{15}$N(-C$<em>{20}$H$</em>{26}$O$_{3}$)SVAR (+3)</td>
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<tr>
<td>NIA</td>
<td>628.37</td>
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Casey Charvet, Wei-Li Liao, Gun Young Heo, James Laird, Robert G. Salomon, Illarion V. Turko and Irina A. Pikuleva

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