Abstract

Posttranslational modification by isolevuglandins (isoLGs), arachidonate oxidation products, is an important yet understudied process associated with altered protein properties. This type of modification is detected in cytochrome P450 27A1 (CYP27A1), a multifunction enzyme expressed in almost every cell and involved in the metabolism of cholesterol and other sterols. Previously, the CYP27A1 Lys358-isoLG adduct was found in human retina afflicted with age-related macular degeneration. Yet, the effect of Lys 358 modification on enzyme activity was not investigated. Herein, we characterized catalytic properties of Lys 358 as well as Lys 476 CYP27A1 mutants before and after isoLG treatment and quantified the extent of modification by multiple reaction monitoring. The K358R mutant was less susceptible to isoLG-induced loss of catalytic activity than the wild type (WT), whereas the K476R mutant was nearly as vulnerable as the WT. Both mutants showed less isoLG modification than WT. Thus, modification of Lys 358 , a residue involved in redox partner interactions, is the major contributor to isoLG-associated loss of CYP27A1 activity.

Our data show the specificity of isoLG modification, provide direct evidence that isoLG addition impairs enzyme activity, and support our hypothesis that isoLG modification in the retina is detrimental to CYP27A1 enzyme activity, potentially disrupting cholesterol homeostasis.

Cytochrome P450 27A1 (CYP27A1) is a ubiquitously expressed mitochondrial enzyme metabolizing a number of important endogenous compounds [reviewed in (1)]. In extraocular tissues, the functions of CYP27A1 include the conversion of cholesterol to 27-hydroxyl and 27-carboxyl oxysterols (1, 2), bioactivation of vitamin D3 (3), C27 hydroxylation of bile acid intermediates (4, 5), and metabolism of the toxic 7-ketocholesterol (6–8). The 27-oxygenated cholesterol metabolites may also play a regulatory role in some organs (9–12). Accordingly, patients with cerebrotendinous xanthomatosis, a rare lipid storage disease caused by inborn errors in CYP27A1, suffer from bile acid deficiency, cholestanol-containing brain and tendon xanthomas, neurological dysfunctions, premature atherosclerosis, and sometimes osteoporosis (13). The role of CYP27A1 in the eye is still under investigation, but available evidence indicates the importance of this enzyme for normal ocular function. Patients with cerebrotendinous xanthomatosis also experience a variety of ocular symptoms including cholesterol exudates and premature retinal senescence (14–16). In addition, they develop juvenile bilateral cataracts. We found that CYP27A1 is the major retinal cholesterol hydroxylase (17–19), also participating in the elimination of 7-ketocholesterol (20, 21), suggested to play a role in the development of age-related macular degeneration (AMD) (22). In addition, we established that Cyp27a1-null mice have focal retinal cholesterol deposits coincident with abnormal vascularization (21).
While genetic abrogation of CYP27A1 is rare, we hypothesized that aging, which is associated with increased oxidative stress, could reduce the activities of CYP27A1, thus leading to pathologies in the eye (23). This hypothesis was based on our observation that there was no correlation between CYP27A1 expression and CYP27A1 product levels in the retina, suggesting the existence of a retina-specific posttranslational modification affecting enzyme activity (17). Furthermore, a CYP27A1 tryptic peptide, VVLAPETGELK \(^{476}\), was consistently underrepresented in the quantitative mass spectrometry data (17). Hence, we turned our attention to posttranslational modification with isolevuglandins (isoLGs), a family of extremely reactive \(\gamma\)-ketoaldehyde derivatives, formed endogenously through enzyme or free radical-mediated oxidation of arachidonic acid (24, 25). IsoLGs have been shown to posttranslationally modify free primary amino groups present in biomolecules with an avidity greatly exceeding other reactive oxidized lipids (26, 27). Targets of isoLG modification include proteins and phospholipids (PLs) in the serum, the vasculature, and the eye, forming stable covalent adducts with isoLGs (26–29). IsoLG adduct levels have been shown to be a biomarker of oxidative stress, increased in patients with atherosclerosis, renal disease, and AMD (28, 30, 31).

The retina is a relevant organ in which to look for posttranslational modification with isoLGs because it contains high quantities of arachidonic acid and has a highly oxidative environment (32, 33). Furthermore, retinal proteins were shown to be subjected to modification with the oxidation products of docosahexaenoic acid (34), another polyunsaturated fatty acid abundant in the retina (35), and isoLG PL adducts are at higher concentrations in plasma of patients with AMD than in healthy volunteers (28). Mass spectrometry analysis of human retina afflicated by AMD revealed that CYP27A1 is indeed posttranslationally modified by isoLGs and identified Lys \(^{358}\) as one of the affected amino acid residues (23). Treatment of purified recombinant CYP27A1 with authentic iso[4]levuglandin E\(_2\) (iso[4]LGE\(_2\)) led to deterioration of CYP27A1 catalytic activity in vitro and modification of Lys \(^{358}\), as well as other amino acid residues including Lys \(^{476}\) (23). Because covalent iso[4]LGE\(_2\) adducts were found at several locations in the CYP27A1 primary sequence, it was not clear how modification of the individual amino acid residues contributes to the loss of CYP27A1 function. Therefore, in the present work, we used a combination of site-directed mutagenesis, biochemical evaluations, and a simplified multiple reaction monitoring (MRM) assay to quantify total Lys-iso[4]LGE\(_2\) adducts. We report that CYP27A1 Lys \(^{358}\) and Lys \(^{476}\) are highly amenable toward isoLG modification with the addition at Lys \(^{358}\) being the underlying reason for the decreased CYP27A1 activity following isoLG treatment. This is the first demonstration of the specificity of isoLG modification and its direct effect on enzyme activity, thus strengthening and providing additional mechanistic insights in our proposed schema (23) whereby increased oxidative stress deleteriously affects protein function and contributes to age-related cholesterol accumulation in the retina and associated vascular pathologies.

MATERIALS AND METHODS

Materials

Authentic iso[4]LGE\(_2\) was synthesized as described and assayed by NMR (36). This compound was reacted with L-Lys to produce Lys-iso[4]LGE\(_2\) adducts (37) which were characterized by ESI-MS/MS. The \(-\gamma\)-butyroxy-carbonyl-pentadecapeptide VVLAPETGELKSVAR\(^{480}\), based on the CYP27A1 Lys \(^{476}\)-containing tryptic peptide, was custom synthesized by American Peptide Co., Inc. (Sunnyvale, CA). Synthesis and characterization of \([\text{H}]_{3}\)iso[4]LGE\(_2\) and preparation and purification of the VVLAPETGELK-(iso[4]LGE\(_2\) lactam)SVAR\(^{390}\) and VVLAPETGELK-(\([\text{H}]_{3}\)iso[4]LGE\(_2\) lactam)SVAR\(^{360}\) internal standard (d\(_6\)-IS) will be published elsewhere. Aminopeptidase M and pronase were from EMD Millipore (Billerica, MA). Oligonucleotides for site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). 1,2-Dilauroyl-sphingo-3-phosphocholine (DLPC) was from Avanti Polar Lipids (Alabaster, AL). Mitochondrial PLs from bovine retina were isolated as described (23). The QuikChange site-directed mutagenesis kit was from Agilent Technologies (Santa Clara, CA). The pGro7 plasmid was part of the Chaperone Plasmid Set from Takara Bio (Mountain View, CA). Gene Choice Escherichia coli strain GC5 competent cells were purchased from Geneseed Scientific (San Diego, CA). Sequencing grade trypsin was from Promega Corp. (Madison, WI). \([1,2-\text{H}\_4\text{N}]\) cholesterol was purchased from Perkin Elmer (Waltham, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC grade.

Preparation of CYP27A1 Lys \(^{358}\) and Lys \(^{476}\) mutants

Mutagenic oligonucleotides are documented in supplementary Table I. Mutations were introduced to the wild-type (WT) CYP27A1 using the QuickChange site-directed mutagenesis kit according to the manufacturer’s instructions. The entire CYP27A1 coding region of the expression constructs was sequenced to confirm generation of the desired mutations and absence of undesired mutations. E. coli strain GC5 was cotransformed with either CYP27A1 WT or a mutant plasmid and with the pGro7 plasmid encoding the groES/groEL chaperone by heat-shock according to the manufacturer’s instructions. Subsequent P450 expression and purification were as described (38) but omitted the chromatographic step on the adrenodoxin-sepharose column. All mutants had only one peak, at 450 nm, in the CO-reduced difference spectrum indicating a lack of denatured P420 protein (39). Protein concentrations were calculated using an absorption coefficient of 91 m\(^{-1}\)cm\(^{-1}\) between \(A_{450}\) and \(A_{430}\) (39). Mutations were also confirmed at the protein level by LC-MS/MS peptide mapping after CYP27A1 carboxidimethylation, reduction, and in-solution trypsin digest as described (23). MS\(^{+}\) peak lists were generated from the data by Mascot Daemon and submitted to the Mascot search engine, version 2.3.0 (Matrix Science). S-carboxidimethylation of Cys was set as a fixed modification and oxidation of Met (Met sulfoxide) was set as a variable modification. Peptide mapping searches used our custom database containing the sequences for mature human CYP27A1 and the K358L/R and K476L/R mutants.

Measurement of catalytic activity

Measurement of the CYP27A1 catalytic activity was based on the conversion of cholesterol to 27-hydroxycholesterol as described (23, 40). The in vitro reconstituted system contained 40 pmol P450, 1,200 pmol adrenodoxin, 160 pmol adrenodoxin reductase, 1 mM NADPH, 20 \(\mu\)M cholesterol, 11 pmol \([\text{H}]\text{cholesterol}, 0.09\%\) 2-hydroxypropyl-\(\beta\)-cyclodextrin (HPCD), and optionally PL vesicles (10 \(\mu\)g/mL, prepared either from DLPC or PLs isolated from retinal mitochondria). Because cholesterol was at a saturating
concentration and product formation was linear with time and protein, the data obtained represent turnover numbers.

**Treatment with iso[4]LGE₂**

CYP27A1 WT and mutants were reconstituted into PL vesicles prepared from bovine retinal mitochondria followed by treatment with iso[4]LGE₂ as described (25). A 24-fold molar excess of iso[4]LGE₂ over a total P450 heme residue content was used. At different time points, aliquots of the reaction mixture were withdrawn and quenched with a 24-fold molar excess (over iso[4]LGE₂) of buffered glycine (100 mM in 50 mM potassium phosphate (KP) buffer, pH 7.2). Time 0 represents a time point immediately prior to the addition of iso[4]LGE₂. Control samples containing no iso[4]LGE₂ were handled identically. For each aliquot, the CO-reduced difference spectrum and CYP27A1 catalytic activity were measured.

**Optimization of mass spectrometry parameters for the measurement of Lys-iso[4]LGE₂ lactam adducts**

To tune the quadrupole parameters and confirm that the previously reported Lys-iso[4]LGE₂ lactam fragment ions with m/z 84 and 332 (41) were indeed generated by our mass spectrometer from the precursor ion m/z 479, we prepared Lys-iso[4]LGE₂ from authentic materials. The adduct mixture (approximately 5 μg/ml in 30% acetonitrile (ACN) in water containing 0.1% formic acid) was infused at a rate of 10 μl/min directly into the ESI source of the hybrid triple quadrupole/linear ion trap (LIT) mass spectrometer (4000 QTrap, AB SCIEX, Framingham, MA) operating in the positive ionization mode with the following source parameters: an ion spray voltage of 5,500 V, curtain gas of 15 psi, ion source gas of 30 psi, heater gas of 60 psi, and interface heating temperature of 200°C. MS² data for the m/z 479 precursor ion were collected in the product ion scan mode with the third quadrupole (Q3) operating as a resolving quadrupole with a scan time of 5 s. The parameters were set to maximize the signal-to-noise ratio of the m/z 84 product ion: declustering potential of 50 V, entrance lens potential of 10 V, collision energy of 55 eV, and collision cell exit potential of 4 V. The resultant MS² spectra for the product ion scan of the m/z 479 precursor ion using these parameters is given in supplementary Fig. I. These parameters were also optimal for generation of the m/z 322 product ion and thus were used for all transitions in the MRM assay.

**LC-MS/MS analysis of the VVLAPETGELK-(iso[4]LGE₂ lactam)SVAR₄₈₀ peptide**

To aid in development of the quantitative MRM workflow the VVLAPETGELK-(iso[4]LGE₂ lactam)SVAR₄₈₀ peptide was digested to release the Lys-iso[4]LGE₂ lactam adduct to then be analyzed by LC-MS/MS (see next section for sample processing and LC-MS/MS conditions). MS² data for the m/z 479 precursor ion were collected in the enhanced product ion scan mode with Q3 operating in LIT mode to generate higher quality MS² spectra. The total ion current chromatogram from this product ion scan revealed two minor peaks eluting at 20.2 and 21.9 min (supplementary Fig. II, peaks A, B) and two major peaks eluting at 22.4 min and 25.1 min (supplementary Fig. II, peaks C, D). The MS² spectrum of peak A (supplementary Fig. II A) shared little in common with those of peaks B–D, and was likely not Lys-iso[4]LGE₂ lactam. Peak B had an MS² spectrum with fragment ions at m/z 132, 247, 265, 311, 332, 370, 397, 415, 443, and 461 (supplementary Fig. II B), similar to the MS² spectra of the two major peaks (supplementary Fig. II C, D) except for m/z 132 and 370. Lys-iso[4]LGE₂ has two stereocenters (supplementary Fig. II, inset), in addition to the all-L absolute configuration of the lysyl group. Thus, it is expected to generate four diastereomers because the isoLG is racemic and the adduct formation is nonstereoselective. The identical spectra of the two major peaks in supplementary Fig. II suggests resolvable diastereomers. Hence, all subsequent calculations of the extent of isoLG modification of CYP27A1 were based on the combined intensities from the two major peaks C and D. We also noted that the m/z 84 product ion was not observed in the spectra produced in the LIT mode while it was very abundant in MRM and quadrupole scanning modes.

**Quantification of total Lys-iso[4]LGE₂ lactam adducts in CYP27A1**

To minimize variability, all samples were processed simultaneously using a simplified quantitative MRM workflow for the detection of isoLG lactam adducts that we developed based on previously published methods (37, 42). We diluted 100 pmol of iso[4]LGE₂-treated CYP27A1 7.5-fold with 25 mM NH₄HCO₃ containing 1% SDS to a final volume of 150 μl followed by heating to 90°C for 5 min. Samples were then sequentially mixed with 4 vol of methanol, 1 vol of chloroform, and 3 vol of water (45). The sample was then centrifuged at 17,000 g for 10 min to precipitate the protein in the form of the disc at the interface between the aqueous and organic phases. Both phases were then aspirated, eliminating salts and lipids from the protein sample. The protein precipitate was supplemented with 250 pmol of d₆-IS and resuspended in 100 μl 25 mM NH₄HCO₃ containing 10 mM CaCl₂ by sonication (15 × 1 s pulses, 20% cycle, 10% power) with a Digital Sonifier S-450D (Branson Ultrasonics, Danbury, CT). The mixture was digested with 0.4 μg pronase (~1:1, mg protease/mg total protein) at 37°C for 24 h, then supplemented with 2 mM MgCl₂ and digested with 0.0026 U aminopeptidase M (1:15, units protease/mg total protein) at 37°C for 18 h. During digestion, the mixture was subjected to constant shaking. The digest was then centrifuged at 17,000 g for 10 min to spin down undigested protein and insoluble material. The supernatant was transferred to a clean tube and evaporated to dryness in a Savant SC210A SpeedVac concentrator (Thermo Scientific, Asheville, NC). Samples were stored at −80°C and when analyzed dissolved in 50 μl 5% ACN in water containing 0.1% formic acid. Samples were resolved on an Ultimate 3000 LC system (Dionex, Sunnyvale, CA) with a Zorbax Eclipse Plus C₁₈ column (2.1 × 100 mm, 3.5 μm, Agilent) with matching guard column maintained at 25°C with a gradient from 5% to 60% ACN in water with 0.1% formic acid over 40 min at a flow rate of 200 μl/min. A switching valve was used to initially divert the eluent to waste for the first 5 min of the gradient. Then the switching valve directed the column effluent into the 4000 QTrap MS ESI source operating in the positive ionization mode, MRM chromatograms of the two transitions for Lys-iso[4]LGE₂ lactam and the corresponding two transitions for the deuterium labeled internal standard were acquired using the optimized parameters listed above. Because of the higher signal-to-noise ratio, the m/z 479→84 and m/z 485→484 transitions were used for quantification, while the m/z 479→332 and m/z 485→338 transitions served as a qualifier to confirm the identity of the peaks as Lys-iso[4]LGE₂ lactam adducts. To assess the extent of modification, a calibration plot was generated using a fixed concentration of the d₆-IS and varying concentrations of the unlabeled VVLAPETGELK-(iso[4]LGE₂ lactam)SVAR peptide in the presence of unmodified WT CYP27A1. The data obtained were fit using linear regression. The extent of Lys-isoLG adduction in treated proteins was then calculated according to the formula: (250 pmol d₆-IS/100 pmol P450) × (Aₐ₆/₅₄₇₀₉₄₆₅₈₂ × Aₐ₆/₅₄₇₀₉₄₆₅₈₂), where A represents the integrated peak areas and 0.732 is the coefficient obtained from the calibration
curve. To check for variability in enzymatic digest, two aliquots from the same preparation of modified CYP27A1 WT and mutants were independently digested and analyzed.

Spectral studies
CYP27A1 WT and mutants were titrated with sterols as described (44) in the absence or presence of DLPC (40 μg/ml). Cholesterol was added from 1 mM stock in 4.5% HPCD and cholesterol-3-sulfate (C3S) was added from 1 mM and 5 mM stocks in 4.5% HPCD. The P450 solution (0.4 μM in 50 mM KPO4, pH 7.2) contained 0.5 M NaCl, 1 mM EDTA, and 10% glycerol and was maintained at 30°C. Ligand-induced changes in the spectra of the P450s were recorded with a UV-2450PC dual beam spectrophotometer (Shimadzu, Japan).

Studies of P450 subcellular distribution
Membrane binding properties of the CYP27A1 WT and mutants were evaluated by isolation of the E. coli membranes after heterologous P450 expression as described (45). The P450 content was measured in the whole homogenate, as well as the cytosolic, and membrane fractions after centrifugation of the whole homogenate at 106,000 g for 90 min. The concentration of the P450 was determined from the CO-reduced difference spectrum (39).

Computational modeling of CYP27A1
CYP27A1 tertiary structure was modeled using the Phyre² server (46) in the one-to-one threading mode. The crystal structure of bovine CYP11A1 in complex with 22-hydroxycholesterol, PDB 3MZS (47), was used as the template (29% of amino acid identity with human CYP27A1) with a reported confidence of 100%. The active site cavity was calculated using VOIDOO (48) as described (49).

Statistical analysis
The enzyme activity data following iso[4]LGE₂ treatment were analyzed using a one-way repeated measures ANOVA (SAS/STAT, SAS Institute, Inc.). P values of less than 0.05 were considered significant.

RESULTS
Effect of mutations of Lys 358 and Lys 476 on catalytic properties of CYP27A1 in the presence of DLPC
IsoLG modification of protein lysyl residues incorporates their ε-amino group into a pyrrole ring in the formed adduct. This decreases the availability of the nitrogen lone pair of electrons for protonation at neutral pH resulting in a loss of the positive charge usually associated with protein lysyl residues under physiological conditions. Hence, CYP27A1 Lys 358 and Lys 476 were mutated to either hydrophobic Leu to mimic removal of the positive charge at these positions or to Arg to conserve the charge but abolish iso[4]LGE₂ modification because Arg is at least 1,000-fold less susceptible than Lys to modification by isoLGs (50). In the presence of DLPC, the K358R CYP27A1 mutant had activity similar to the WT, whereas the K358L mutant was essentially catalytically inactive (Table 1). Likewise, the K476R mutation did not affect the catalytic properties of CYP27A1, in contrast to the K476L substitution which led to almost complete loss of enzymatic activity.

Effects of iso[4]LGE₂ on the activity of CYP27A1 WT and mutants
Because the K358L and K476L mutants were catalytically inactive, only the WT and K358R and K476R mutants were treated with iso[4]LGE₂ (Fig. 1A). After 30 s of iso[4]LGE₂ treatment, the K358R mutant retained 82% of activity relative to the untreated control, whereas the K476R mutant and WT retained less than 50% of activity. Within the next 120 min, activity of the K358R mutant further declined by 29% while that of the K476R mutant and WT declined by 13% and 25%, respectively. Thus, the K476R mutant, where modification can occur at residue 358 and not 476, had a loss of enzyme activity similar to the WT where modification can occur at both residues 358 and 476 simultaneously. In contrast, by preventing modification at residue 358, the K358R mutation rendered the CYP27A1 less susceptible to loss of enzyme activity from iso[4]LGE₂ treatment. This result suggests that isoLG modification of CYP27A1 at residue 358 is more detrimental to enzyme activity than modification at position 476.

The extent of iso[4]LGE₂ modification of CYP27A1 WT and mutants
The MRM assay of CYP27A1 samples at time 0, i.e., prior to iso[4]LGE₂ treatment, showed no evidence of Lys[1H₆]iso[4]LGE₂ lactam adducts with only Lys-[1H₆]iso[4]LGE₂ lactam from the internal standard present (Fig. 2A, A’). However, peaks corresponding to unlabeled Lys[iso[4]LGE₂ lactam were seen at 30 s (Fig. 2B, B’) and 5 min (Fig. 2C, C’) of treatment. For the WT, the total number of Lys residues modified after 30 s of treatment was 8.1–8.5 and increased slightly to 8.6–8.9 after 5 min of treatment (Fig. 1B). This time course of modification corresponded to a sharp loss of enzymatic activity occurring after 30 s of iso[4]LGE₂ treatment (~50%, Fig. 1A) and a moderate additional loss in CYP27A1 activity within the next 2 h of modification (~25%). Perhaps modifications other than lactam formation (e.g., protein cross-linking (51)) begin to occur in CYP27A1 after 15 min of treatment, and these modifications contribute to further loss of enzyme activity. Consistent with unavailability of Lys[1H₆] for isoLG ad-duction, the K476R mutant had fewer residues modified than the WT at both time points investigated but demonstrated similar kinetics of isoLG modification: 5.2–5.3 lysyl residues after 30 s and a slightly higher degree of modification, 5.6–6.1 lysyl residues, after 5 min. Although fewer residues were modified, decline in enzyme activity was similar to that of the

|                | Turnover (min⁻¹) * |
|----------------|-------------------|
| CYP27A1        |                   |
| WT             | 10 ± 2            |
| K358R          | 10 ± 1            |
| K358L          | <0.5              |
| K476R          | 10 ± 4            |
| K476L          | <0.5              |

Values are the mean of triplicate measurements ± SD.

CYP27A1 enzymatic activity was measured in the reconstituted system in vitro as described in Materials and Methods.

Table 1. Effect of mutation of Lys 358 and Lys 476 on CYP27A1 activity in the presence and absence of DLPC-containing PL vesicles
WT at all time points, indicating that modification of Lys\(^{376}\) does not account for the major loss of enzyme activity seen in the WT. The kinetics of the isoLG modification of the K358R mutant was different: 5.0–5.5 lysyl residues after 30 s of treatment, fewer than in the WT but similar to the number in the K476R mutant, and additional modifications, 7.6–7.9 lysyl residues in total, occurring after 5 min of treatment. This continuous isoLG modification in the K358R mutant corresponded to a smaller initial loss of enzyme activity after 5 s as compared with the WT and K476R mutant followed by a more significant further decline within 2 h. Perhaps, modification of K476 in the K358R mutant exposes other lysyl residues for interaction with isoLG, and these residues are important for CYP27A1 catalytic function.

**Functional role of Lys\(^{376}\)**

Reductions in the enzymatic activity of the CYP27A1 WT and K476R mutant upon isoLG treatment were similar and higher than the catalytic changes in the K358R mutant (Fig. 1A). This suggests the importance of a positive charge at position 358 and is consistent with the known role of Lys\(^{388}\) as a residue involved in the electrostatic interactions with the

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**Fig. 1.** Effect of iso[4]LGE\(_2\) treatment on the enzymatic activity of CYP27A1 WT and mutants (A) and extent of modification (B). Properties of the CYP27A1 WT are shown in gray, and those of the K358R and K476R mutants are in blue and red, respectively. Enzyme activity data are expressed as a percentage relative to untreated controls and values are the mean ± SD from three independent experiments in which the P450s were treated with the same preparation of iso[4]LGE\(_2\). A statistically significant difference in remaining activity over the course of isoLG treatment was found only for the K358R mutant as compared with the WT (\(P < 0.03\)) and the K476R mutant (\(P < 0.04\)). Values for the number of Lys-iso[4]LGE\(_2\) lactam adducts are from two independent MRM assays of samples modified with the same preparation of iso[4]LGE\(_2\). Time on the x axis is plotted in logarithmic scale.

**Fig. 2.** Representative MRM chromatograms of the proteolytic digest of CYP27A1 WT before (A, A’) and after iso[4]LGE\(_2\) treatment for 30 s (B, B’) and 5 min (C, C’). The two transitions corresponding to the deuterated internal standard (Lys-[\(^{2}\)H\(_6\)]iso[4]LGE\(_2\)) are shown in gold and magenta, while those from iso[4]LGE\(_2\)-modified CYP27A1 are shown in blue and green. Each pair of panels (e.g., A and A’) are from a single MRM acquisition in which the 4 transitions were monitored simultaneously.
P450 redox partner adrenodoxin (52). The role of Lys476, however, has not yet been elucidated, and it is not clear how the Lys476-isolG adduction could lead to modification of other lysyl residues in CYP27A1. Therefore, we extended the evaluation of the properties of the K476R/L mutants beyond the measurements of their catalytic activities and generated a computational model of CYP27A1 to serve as initial guidance in these evaluations (Fig. 3). This model was based on the crystal structure of CYP11A1 (47), which like CYP27A1 is a mitochondrial enzyme metabolizing the same substrate (cholesterol) and interacting with the same redox partner (adrenodoxin). The CYP27A1 model placed Lys476 on the surface of the protein in the β4-1 sheet region, which forms a part of the enzyme active site. Yet, the side chain of Lys476 in this model extends into the solution rather than inside the substrate binding cavity suggesting that Lys476 is unlikely to be involved in direct interactions with cholesterol. Accordingly, the inactivating effect of the K476L mutation could be due to the indirect effect of the mutation on protein conformation which in turn affects cholesterol binding. To test this inference, we used a spectral binding assay and titrated the K476L and K476R mutants with cholesterol (Table 2) in the buffer lacking DLPC because PL vesicles may increase the solution light scattering. The spectral response of the K476L mutant was too weak, precluding reliable determination of the cholesterol $K_d$. In contrast, the K476R mutant showed the same type of cholesterol-induced spectral shifts as the WT and had an apparent $K_d$ for cholesterol of 1.1 μM, similar to that of the WT (0.6 μM). To model the conditions of the enzyme assay, CYP27A1 WT and Lys476 mutants were titrated with cholesterol again following P450 reconstitution in DLPC vesicles. The cholesterol apparent $K_d$ for the K476R mutant became 4-fold higher than that for the WT because the presence of DLPC decreased the $K_d$ for the WT 2-fold and did not affect the $K_d$ for the K476R mutant. Perhaps addition of DLPC causes significant partitioning of cholesterol from the aqueous solution into PL vesicles and increases cholesterol concentration in the lipid bilayer, the site of substrate recruitment to the active site (45). This increase in the effective cholesterol concentration affects the $K_d$ for the WT but is not sufficient to alter the $K_d$ for the K476R mutant.

Next, we tested another CYP27A1 substrate C3S (53) for the ability to elicit spectral changes in the K476L mutant (Table 2). In the absence of DLPC, the C3S-induced spectral response in the K476L CYP27A1 was similar to that of the WT, enabling determination of the sterol’s $K_d$ that was 2.8-fold higher than that of the WT (1.1 μM vs 0.4 μM). The $K_d$ for the K476R mutant was 3.5-fold higher than that for the WT. Addition of DLPC led to a 24-fold increase in the $K_d$ for the WT and 5- and 2.5-fold increases in the $K_d$ values for the K476L and K476R mutants. Accordingly, the apparent $K_d$ values for the K476L and K476R mutants became 6.9- and 4.4-fold higher than that for the WT. C3S is more polar than cholesterol and probably has less affinity for the lipid bilayer leading to the sterol distribution between the aqueous solution and PL vesicles. This decreases the effective concentration of C3S and increases its $K_d$ for both CYP27A1 WT and mutants. Incorporation of CYP27A1 into DLPC vesicles could also stabilize the altered conformation of the K476L and K476R mutants, thus explaining why the values of the C3S $K_d$ for the mutants are higher in the presence of DLPC than in the absence of DLPC.

The effect of DLPC on CYP27A1 substrate binding prompted us to investigate if mutation of Lys476 alters the interactions of CYP27A1 with the membrane as assessed by quantifications of the P450 subcellular distribution in E. coli. Analysis of the P450 content in the E. coli membranes and cytosol showed that all P450s are bound to the membrane fraction and with none detected in the cytosol (supplementary Table II).

We also evaluated the effect of DLPC on CYP27A1-mediated cholesterol hydroxylation (Table 1). Absence of DLPC decreased the turnover numbers of the WT and K476R mutant, consistent with our interpretation that PL vesicles concentrate cholesterol and increase its effective concentration. Because cholesterol in the enzyme assay was at a saturating concentration, differences in the $K_d$ of WT and K476R did not affect the turnover number. The effect on the activity of the K476L mutant was the opposite as omission of DLPC led to quantifiable product formation with the turnover number being four times lower than that of the WT. This result is also consistent with our other explanation that binding to PL vesicle stabilizes the altered conformation of the Lys476 mutant K476L and enhances the effect of the mutation. Collectively, the data obtained support our initial inference from the CYP27A1 model that Lys476 mutation affects the enzyme function indirectly by introducing changes in the protein conformation. The effects are lesser in the case of the conservative K476R substitution and more pronounced in the case of the nonconservative K476L substitution. Hence, similar to the nonconservative K476L mutation, modification of Lys476 by iso[4]LEGe in the K358R mutant could lead to conformational changes exposing more lysyl residues to modification.

**DISCUSSION**

In the present work, we provide direct evidence that the isolG adduction of Lys476 in CYP27A1, as observed in the retina of an AMD afflicted donor (23), indeed impairs...
enzyme activity. IsoLG-mediated loss of enzyme activity has been reported before (29, 41, 54–56), however, the evidence provided were indirect. In the pioneering study of Fukuda et al. (54), isoLG-treated HEK-295 cells and cultured atrial HL-1 myocytes showed reduced electrophysiology recordings, but isoLG modification of the cardiac sodium channels responsible for this dysfunction was not shown. In another study, the enzyme activity of purified RNase A and glutathione reductase declined upon treatment with isoLG in vitro, and inclusion of an isoLG scavenger in the reaction mixture was able to prevent this loss of activity (41). However, mechanisms underlying this loss were not investigated further. The first evidence linking isoLG modification and loss of enzyme activity in vivo was for the protease calpain-1. The levels of this enzyme are elevated in glaucomatous astrocytes (55) and trabecular meshwork (29) as compared with nonglaucomatous controls. Nevertheless, despite elevated levels, calpain-1 activity is reduced as revealed by an ex vivo assay (29). In the same study, treatment of purified calpain-1 with isoLG in vitro resulted in loss of catalytic activity but specific sites of modification were not reported. Lastly, in a study assessing the effect of isoLG treatment on isolated mitochondria, the treated mitochondria showed impaired respiration related to complex III (56). The respiration defect was resolved by adding purified cytochrome c to the isoLG treated mitochondria, but it was not apparent how isoLG treatment affected cytochrome c. When purified cytochrome c was then treated with isoLG in vitro, modification was localized by mass spectrometry to the lysyl rich N-terminal region, although not to a specific residue.

In contrast to all previous studies (29, 41, 54–56), we seem to be the first to conduct the measurement of enzyme activity and simultaneously correlate the isoLG modification to specific locations in the primary sequence. The data obtained support our previous findings that LysisoLG and Lys isoLG are both targets of isoLG adduction in CYP27A1 (23). Our results also strengthen interpretations from other reports on isoLG-associated loss of enzyme activity (29, 41, 54–56) and demonstrate that posttranslational protein modification by isoLGs is not simply a biomarker but is in fact a mechanism by which oxidation of lipids leads to modulation of enzyme activity. It should be noted, then, that in retinal mitochondria, the targets for isoLG adduction may not be limited to CYP27A1. Other cholesterol-related proteins could be modified as well and affect cholesterol metabolism in the retina. The importance of the present work is that it fills a gap in our putative scheme (Fig. 4) linking oxidative stress and disrupted cholesterol homeostasis which eventually leads to pathologies, e.g., abnormal vascularization in the retina and formation of atheromas in the blood vessels of the systemic circulation. Accordingly, we propose that inhibition of isoLG modification could ameliorate this negative aspect of oxidative stress. The investigation of pharmacologic treatments preventing isoLG modification and its deleterious effect on the retina in vivo is in progress in this laboratory.

Another novel aspect of our work is the development of a refined MRM assay to measure the extent of Lys isoLG modification in proteins based on analysis of Lys isoLG lactam adducts (37, 42, 54). We implemented the following changes. First, prior to peptide hydrolysis, samples were heated in 1% SDS to completely unfold higher order protein structures to improve the yield of protein digest. Second, SDS, salts, and lipids were removed through chloroform/aqueous methanol extraction while precipitating the protein. Third, we eliminated the step of base hydrolysis of fatty acid esters. This step is important in biological samples where isoLGs can be generated from arachidonate esterified to PLs, but it is not necessary for in vitro studies in which authentic isoLGs are used to modify proteins. Fourth, we utilized stable isotope labeled [1H6]iso[4]LGE2 to generate our internal standard. This is in contrast to previously published protocols which used a standard consisting of a mixture of radioisotope labeled [1H6]Lys and stable isotope labeled [15C6,15N2]Lys reacted with isoLGE2. Our standard negates the additional precautions needed for handling radioactive material and resultant waste and avoids the introduction of radioactive material into the mass spectrometer. Fifth, the standard used in previous protocols was added prior to sample analysis and does not account for the efficiency of the tandem enzymatic digestion. We synthesized an internal standard in the form of a pentadecapeptide and added it prior to digestion thus accounting for and minimizing variability from peptidase digest efficiency.

By applying our MRM assay to analyze the extent of isoLG adduction in the WT CYP27A1, we established that when a 46-fold molar excess of iso[4]LGE2 over the protein was used, only an average of 8.6 to 8.9 out of 23 lysyl residues were modified after 5 min. It is unlikely that in vivo, isoLGs would accumulate to a large excess over total protein. Hence, only the most reactive lysyl residues will be prone to modification with susceptibility probably depending on spatial access to the lysyl residues and their local environment, e.g., absence of electrostatic and hydrogen-bonding interactions with other amino acid residues. Despite similar reactivity, the

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**TABLE 2.** Effect of mutation of Lys476 on CYP27A1 binding of cholesterol and C3S in the absence and presence of PLs.

|                | Cholesterol | C3S | Cholesterol | C3S |
|----------------|-------------|-----|-------------|-----|
|                | $K_i$ (μM)  |     | $\Delta A_{max}$ (μmol P450) |
| CYP27A1        |             |     |             |     |
| WT             | 0.6 ± 0.2   | 0.3 ± 0.1 | 0.4 ± 0.2 | 0.8 ± 0.1 |
| K476L          | LSR         | LSR | 1.1 ± 0.1   | 1.4 ± 0.1 |
| K476R          | 1.1 ± 0.1   | 1.2 ± 0.1 | 3.5 ± 0.1   | 3.5 ± 0.1 |

LSR, low spectral response. Values are the mean ± SD of triplicate measurements.
functional role of lysyl residues could be different as indicated by our studies. This implies that proteins with even a low level of overall modification could have significantly impaired enzymatic properties. As isoLGs are representative members of a large family of reactive aldehydes generated in human tissues, e.g., 4-hydroxynonenal, malondialdehyde, and hydroxy-ω-oxoalkenonoic acids, the isoLG reactive lysines could also be subjected to other types of modification.

Site-directed mutagenesis is a widely used approach in the study of different types of posttranslational modification, e.g., Tyr to Phe mutations to prevent phosphorylation (57) and Lys to Arg mutations to prevent ubiquitination (58), sumoylation (59), and acetylation (60). However, Lys mutants have never been studied for covalent addition by isoLGs. Our data indicate that site-directed mutagenesis is indeed effective in reducing total isoLG modification of lysyl residues, and in the case of the K358R mutant, reducing the degree of isoLG-mediated loss of enzyme activity.

In summary, we confirmed that iso[4]LGE2 modification of CYP27A1 at Lys358 and Lys476 negatively affects enzyme catalytic activity and showed that prevention of modification at residue 358 through the charge-conservative mutation of Lys to Arg reduces isoLG-mediated loss of enzyme function.

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