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Mass Spectrometry Detection of Isolevuglandin Adduction to Specific Protein Residues

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Abstract

The aging process seems to be associated with oxidative stress and hence increased production of lipid peroxidation products, including isolevuglandins (isoLGs). The latter are highly reactive γ -ketoaldehydes which can form covalent adducts with primary amino groups of enzymes and proteins and alter the properties of these biomolecules. Yet, little is currently known about amino acid-containing compounds affected by isoLG modification in different age-related pathological processes. To facilitate the detection of these biomolecules, we developed a strategy in which the purified enzyme (or protein) of interest is first treated with authentic isoLG *in vitro* to evaluate whether it contains reactive lysine residues prone to modification with isoLGs. The data obtained serve as a basis for making the “GO/NO GO” decision as to whether to pursue a further search of this isoLG modification in a biological sample. In this chapter, we describe the conditions for the *in vitro* isoLG modification assay and how to use mass spectrometry to identify the isoLG-modified peptides and amino acid residues. Our studies were carried out on cytochrome P450 27A1, an important metabolic enzyme, and utilized iso[4]levuglandin E₂ as a prototypical isoLG. The isoLG-treated cytochrome P450 was subjected to proteolysis followed by liquid chromatography-tandem mass spectrometry for peptide separation and analysis by Mascot, a proteomics search engine, for the presence of modified peptides. The developed protocol could be applied to characterization of other enzymes/proteins and other types of unconventional post-translational protein modification.

Keywords

isolevuglandin; γ -ketoaldehyde; post-translational modification; mass spectrometry; multiple reaction monitoring; CYP27A1; Mascot

1. Introduction

Isolevuglandins (isoLGs) are a family of extremely reactive γ -aldehydes named for their levulinialdehyde nucleus and prostaglandin-like structure (1). These compounds were discovered in the 1980s and shown initially to form through spontaneous rearrangement of prostaglandin H₂ (2, 3). Later, isoLGs were also found to arise via free radical oxidation of arachidonic acid (1, 4–6). While the rearrangement of prostaglandin H₂ produces only

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levuglandin E₂ (Fig. 1a), the free radical oxidation yields a large array of regio- and stereo-isomers of isoLGs such as iso[4]levuglandin E₂ (iso[4]LGE₂) (Fig 1b) (7). The isoLGs are many times (up to 1000-fold) more reactive than most other lipid oxidation products avidly binding to primary amines in biomolecules and forming different types of adducts (Fig. 1c, d) (5, 6, 8). The existence of these different adducts makes investigation of post-translational modification with isoLGs difficult. Before our work, little was known about which adducts were most abundant. A major benefit of using mass spectrometry (MS) to analyze isoLG adduction is that MS is capable of recognizing and distinguishing these multiple adduct oxidation forms.

IsoLG adducts have been used as a biomarker of long-term oxidative stress. The products of isoLG modification are elevated in the serum of patients with renal disease, atherosclerosis, and age-related macular degeneration (AMD) and also present in a disease-affected tissue such as trabecular meshwork of glaucoma patients and brain of individuals diagnosed with Alzheimer's disease (9–14). Of importance is that isoLG modification may not simply be a biomarker but also a causative agent because of the deleterious impact on the properties of the modified biomolecules (13, 15–17). We were interested in establishing whether a specific enzyme, cytochrome P450 27A1 (CYP27A1), is modified by isoLGs in aged human retina and how this modification affects the enzyme activity (17, 18). CYP27A1 is a ubiquitously expressed mitochondrial sterol 27-hydroxylase which acts on multiple substrates and thereby participates in several metabolic pathways such as cholesterol removal from extrahepatic tissues, bioactivation of vitamin D₃ in the kidney and production of bile acids in the liver (reviewed in (19)). However, the role of CYP27A1 in the retina is not yet fully understood and is currently under investigation in this laboratory (17, 18, 20, 21). To search for CYP27A1-isoLG adducts in human retina, we decided to assess first whether this enzyme could be modified by isoLGs *in vitro* under the conditions when varying isoLG amounts are used and purified recombinant CYP27A1 is reconstituted in phospholipid vehicles that model the enzyme's natural membrane environment (17). The effect of isoLG treatment on enzyme activity was evaluated as well (17, 18). Subsequent MS analysis enabled the identification of the modified CYP27A1 peptides, most reactive lysine residues and most abundant isoLG adducts (17). These results were used for a search of the isoLG-modified CYP27A1 peptides in human retina by multiple reaction monitoring (MRM) (17). MRM is a technique wherein a mass spectrometer fragments the peptides obtained after proteolytic protein digestion and is set up to detect multiple transitions, or pairs of a peptide ion and peptide-fragment ions derived from the fragmentation of this peptide. MRM can unambiguously identify specific peptide modifications in complex biological samples and quantify these modifications with high accuracy (if internal standards are available). MRM, however, requires prior knowledge of the specific sites of modification and structure of the adducts formed. Herein, we describe how to obtain this information from the studies *in vitro*.

2. Materials

2.1. Equipment

1. An Ultimate 3000 LC system was from Dionex (Sunnyvale, CA) with a C₁₈ Acclaim PepMap 100 column (0.075 × 150 mm), also from Dionex.
2. A hybrid Fourier transform ion cyclotron resonance (FTICR)/linear ion trap mass spectrometer, a LTQ FT Ultra, was from Thermo Scientific (San Jose, CA).
3. The Mascot search engine was from Matrix Science (Boston, MA).

2.2. Reagents and Chemicals

1. IsoLGs are not commercially available and must be custom synthesized. We obtained iso[4]LGE₂ from our collaborator Dr. R. Salomon (Case Western Reserve University) (22). A 3 mM stock of iso[4]LGE₂ was prepared in methanol and stored at −20 °C under nitrogen.
2. The protein of interest is either obtained in the laboratory or purchased from a vendor. We heterologously expressed and purified recombinant human CYP27A1 by ourselves as described (23). The protein concentration was 37 μM, and the buffer was 50 mM potassium phosphate (KP_i), pH 7.2, 20% glycerol (see Note 1). To avoid repeated freezing-thawing, the protein was divided into 20 μL aliquots and stored at −80° C.
3. Sequencing grade chymotrypsin and trypsin for proteolytic digestion were from Promega (Madison, WI). The lyophilized enzymes were reconstituted immediately prior to use at 0.1 mg/μL per manufacturer's instructions.
4. Ammonium bicarbonate (50 mM, pH 7.8) and solutions of dithiothreitol (DTT) and iodoacetamide (IAA) on this buffer were prepared immediately prior to use and the excess was discarded (see Note 2).

3. Methods

3.1. Iso[4]LGE₂ treatment of CYP27A1

1. Iso[4]LGE₂ in methanol (1.150 μmol in 383 μL) was dispensed into a glass tube (see Note 3). The methanol was then evaporated with flowing nitrogen.
2. The resulting iso[4]LGE₂ residue was dissolved in 100 μL of 50 mM KP_i, pH 7.2, 100 μM diethylenetriaminepentaacetic acid (DTPA, see Note 4). The solution was vortexed 2 times for 5 s with a 10 min-interval.

¹Buffer composition may vary based on the protein to be modified. However, buffers containing primary amines *e.g.* Tris must be avoided as they will compete with lysine residues to react with iso[4]LGE₂.

²The pH of ammonium bicarbonate upon dissolution is 7.8 and is not adjusted further.

³The amount of iso[4]LGE₂ in this experiment, 1.150 μmol, represented a 10-fold molar excess over the lysine residue content in 5 nmol of CYP27A1, which contains 23 lysine residues per molecule. If such excess of isoLG does not modify a protein of interest *in vitro*, this protein is unlikely to be modified by isoLGs *in vivo* when other proteins are present and the isoLG to protein ratios are much smaller. However, if a high isoLG to protein ratio does lead to isoLG adduction, the next step is to use lower concentration of isoLGs (see Note 11).

⁴DTPA was included in the buffer as an iron chelating agent. Contamination of buffers and glassware with iron could lead to the formation of reactive oxygen species and confound results.

3. The purified CYP27A1, 5 nmol of in 135 μ L, was placed in a separate glass tube and diluted with 765 μ L of 50 mM KP_i , pH 7.2, 100 μ M DTPA.
4. The CYP27A1 solution was transferred to the tube containing the iso[4]LGE₂ solution. The tube was capped and placed in an orbital shaker (30 rpm) at room temperature for 60 min. A sample omitting iso[4]LGE₂ was used as the untreated control.
5. Glycine, 25 μ L of 100 mM stock in 50 mM KP_i , pH 7.2, was added to the reaction mixture to stop CYP27A1 modification with iso[4]LGE₂ by neutralizing unreacted isoLG.
6. The reaction mixture was divided into 100 μ L aliquots and flash-frozen in liquid nitrogen and stored at -80°C .

3.2. Proteolytic Digestion

To maximize protein sequence coverage, we performed two types of digests, in-gel and in-solution, and for each used two types of proteases, trypsin and chymotrypsin.

3.2.1. In-gel digestion—In-gel digestion was followed the standard protocol (24).

1. The sample of iso[4]LGE₂-treated CYP27A1, 15 pmol in 20 μ L, was subjected to 10% SDS-PAGE along with untreated control and molecular weight standards.
2. The gel was stained with Coomassie blue and, for the sample lanes (isoLG treated CYP27A1 and untreated control), the stained region corresponding to proteins with molecular mass between 50 and 60 kDa was excised with razor blade (see Note 5). Each excised band was diced into 1 mm³ cubes and transferred to a separate 1.5 mL microcentrifuge tube.
3. The gel pieces were destained in 100 μ L of 50 mM ammonium bicarbonate/ acetonitrile (1:1 vol/vol) for 30 min with occasional vortexing for 5 s.
4. The gel pieces were dehydrated by the addition of 500 μ L acetonitrile until the pieces became small, hard, and opaque (about 10 min). The liquid was removed by pipetman, taking care not to poke or aspirate the gel pieces (see Note 6).
5. The gel pieces were covered with 100 μ L of 20 mM DTT for 30 min to reduce CYP27A1. The liquid was then removed, and the gel pieces were dehydrated as in step 4.
6. The gel pieces were covered with 100 μ L of 100 mM IAA to alkylate CYP27A1. This procedure was carried out in the dark for 20 min followed by liquid removal, and gel dehydration as in step 4. The gel pieces were air-dried for 5 min.

⁵In our experiments, the iso[4]LGE₂-treated CYP27A1 had slower electrophoretic migration than the untreated control whose molecular weight is ~57kDa.

⁶Gel-loading pipetman tips are ideal for removing or transferring liquid in microfuge tubes with gel pieces. Holding the tip against the bottom of the tube prevents aspiration of gel pieces. Regular tips with wider openings are prone to clogging by aspiration of gel pieces.

7. Chymotrypsin or trypsin was added to the dried gel pieces at a protease to CYP27A1 ratio of 1:50 (w/w) in a final volume of 50 μ L of 50 mM ammonium bicarbonate, pH 7.8. The proteolysis proceeded for 22 h at 37 °C with shaking at 60 rpm.
8. The resultant peptides were extracted by incubating the gel pieces with 100 μ L of 50% acetonitrile containing 5% formic acid for 15 min in a shaker at 37 °C. The tube was centrifuged for 5 sec at 500 rpm to spin down the solution from the cap and sides. The solution was aspirated by pipetman and transferred to new microfuge tube, where it was dried in a vacuum concentrator. The tube was stored at –20 °C until MS analysis.

3.2.2. In-solution digestion—In-solution digestion followed the standard protocol (25).

1. Iso[4]LGE₂-treated CYP27A1, 2 nmol in 400 μ L, was dialyzed overnight against 1000 volumes of 50 mM KP_i, pH 7.2, 100 μ M DTPA, 0.2 M NaCl (see Note 7).
2. The protein sample was concentrated to a volume of 5 μ L in an Amicon Ultracentrifugal filter (regenerated cellulose, 50-kDa molecular mass cutoff, EMD Millipore, Billerica, MA) and transferred to a clean microfuge tube. Crystalline urea (4.8 mg) was added to the protein solution, resulting in a final volume of 10 μ L.
3. DTT (2.5 μ L of 100 mM stock) was added to the protein solution to reduce CYP27A1. The incubation was carried out for 30 min at room temperature. Then IAA (1.5 μ L of 250 mM stock) was added to alkylate the protein for 30 min in the dark. The final volume at this point was 14 μ L.
4. The protein solution was diluted 10-fold with 126 μ L of 10 mM Tris-HCl, pH 8.0, containing 3 μ g chymotrypsin or trypsin at a protease to CYP27A1 ratio of 1:50 (w/w). The protein was digested for 22 h at 37 °C with shaking at 60 rpm (see Note 8).
5. Protein digest (667 pmol in 47 μ L) was applied to an Ultra-Micro C₁₈ PrepTip from the Nest Group (Southborough, MA), and the flow through fraction discarded. Sample loading, washes, and peptide elution were according to the manufacturer's instructions. Acetonitrile (50 μ L of 80% (V/V) aqueous solution) was applied two times to the tip to elute peptides. The eluates were combined in a clean microfuge tube, and dried in a vacuum concentrator. The tube was stored at –20 °C until MS analysis.

3.3. LC-MS/MS Analysis

1. The dried peptides were dissolved in 12 μ L of 2% aqueous acetonitrile containing 0.1% formic acid. One-sixth (2 μ L) of this solution was injected into the LC-MS

⁷Detergent, glycerol, and other additives present in protein solution along with precipitated protein could create problems (e.g., high column pressure and electrospray source clogging) during the subsequent peptide separation by LC and MS/MS analysis.

⁸If the in-solution digest contains protein precipitate, the protease should be added in two portions, a half of the amount to start the digest, and the other half 11–12 hours later. Sonication by microtip at 10% power, 20% duty cycle with a Digital Sonifier S-450D from Branson Ultrasonics (Danbury, CT) may also help disperse larger precipitates and thus facilitate the proteolysis.

system. Peptides were eluted with a 2%–80% gradient of aqueous acetonitrile containing 0.1% formic acid over a 50 min period at a flow rate of 300 nL/min and directed into the nanospray source with the following source parameters: ion spray voltage of 2400 V and an interface capillary heating temperature of 200 °C.

2. Full mass spectra were acquired from the FTICR detector, and the tandem mass spectra (MS/MS) of the eight most intense ions generated by the linear ion trap were recorded in data-dependent acquisition mode (see Note 9) with normalized collision energy of 35 eV, an isolation width of 2.5 Da, and activation Q of 0.25.

3.4. Identification of iso[4]LGE₂ adducts

This section describes how to identify the sites of iso[4]LGE₂ modification from the MS/MS data using the Mascot Search Engine (see Note 10).

1. The set of all possible iso[4]LGE₂ modifications was created by connecting to the university Mascot server. From the welcome page, “Configuration Editor” was clicked, followed by “Modifications”, then the “Add New Modification” button. The “Add Modification” page presents form fields to fill in the title, full name, and composition of the modification. When listing the composition of the iso[4]LGE₂ adduct formula, it is important to subtract 2 hydrogen atoms to account of the two hydrogen atoms that are eliminated from the ε-amino group upon its reaction with iso[4]LGE₂. For example, the lactam adduct has a formula of C₂₀H₃₀O₄, but must be entered into Mascot as C₂₀H₂₈O₄. All 7 modifications (Table 1) were entered in this manner.
2. A new database containing the primary sequence of only CYP27A1 was created by selecting “Configuration Editor” from the welcome page, followed by “Database Maintenance”, to load the “Edit Database Definitions” page. CYP27A1 was entered for “Name”, and the full path and filename to a fasta file containing the primary sequence of mature human CYP27A1 was entered into “Path”. All other options were left as the default.
3. After launching the Mascot Daemon (the application for submitting jobs to the Mascot server), the Parameter Editor tab was selected (Fig. 2). The custom CYP27A1 database was selected in the Database box, and the “Select Modification” button was clicked. “Carbamidomethyl (C)” was selected and added under “Fixed Modification”, while “Oxidation (M)” and the 7 new iso[4]LGE₂ adducts were selected and added under “Variable Modifications”. Either trypsin or chymotrypsin was selected as for the “Enzyme” with “Max missed cleavages” set to 2. The remaining parameters were set as follows: monoisotopic, selected; peptide charge, 2+ and 3+; peptide tolerance, 15 ppm; MS/MS ions search, checked; data

⁹Data-dependent acquisition is an operational mode on Thermo Scientific mass spectrometers capable of MS/MS wherein the software automatically selects the 4 – 8 most intense ions present in the full MS scan for subsequent full MS/MS analysis. Instruments from other vendors with MS/MS capabilities usually offer this mode in their software as well.

¹⁰This section requires creation of a custom database and custom modifications on the Mascot server which is usually installed at universities/companies with mass spectrometry facilities. Please consult with the administrator of your Mascot system if you are not authorized to make changes to the system configuration or are not comfortable with process.

format, Mascot generic, MS/MS tolerance, 0.8 Da; quantitation, none; instrument, ESI-FTICR. The parameter set was saved as “iso4lge2.par”.

4. To submit the MS/MS data files for searching, the Task Editor tab was selected in Mascot Daemon (Fig. 2). The “iso[4]lge2.par” parameter set was loaded by clicking the “...” button under “Parameter Set”. Next, under “Data file list”, the MS/MS file was added. All other parameters were as default and the search was started by clicking “Run.”
5. Mascot identified in the isoLG-treated CYP27A1 MS/MS spectra which corresponded to the enzyme peptides both with and without isoLG modification. Modified residues were underlined in the peptide sequence and the detected modification displayed (Fig 3a). Clicking on a specific query loaded the MS/MS spectra for examination and confirmation of the modification (Fig 3b). The presence of the modified residues in isoLG-treated CYP27A1 and lack of modification in untreated control was used as the basis for the next set of experiments (see Note 11).

3.5. Concluding remarks

The search for unconventional protein modification in a biological sample is extremely difficult if preliminary *in vitro* data on the type of the adduct formed and residue(s) modified are unavailable. Herein we described how to obtain this *in vitro* data and simultaneously gain insight into whether a protein of interest could have this type of modification *in vivo*. Our data indicate that a good pre-requisite for the subsequent analysis of a biological sample is when the protein of interest is amenable to the modification *in vitro* after a short incubation (<1 min) with low molar excesses of the modifying agent (<2-fold). The most reactive lysine residues will also likely be modified in a biological sample (17, 18). Protein location in a cell should also be considered and modeled *in vitro*, e.g. by insertion into liposomes, followed by the modification. If the protein is still amenable to the modification, studies of a biological sample are warranted and should utilize an internal standard to obtain unambiguous results. This internal standard can also be generated based on the information provided by the *in vitro* experiment. In the case of CYP27A1, the most abundant adduct was the isoLG lactam. Thus, collectively, the *in vitro* modification of protein of interest could provide a lot of information for making the “GO/NO GO” decision and essential for successful identification of the unconventional protein modification in a biological sample.

¹¹Identification of the CYP27A1 lysine residues susceptible to modification was a starting point for a number of additional experiments including quantitative MRM. For example, CYP27A1 was incubated with much lower concentration of isoLGs (a 2-fold molar excess over the protein lysine residue content) for 30 sec, 5 min, 15 min, 60 min and 120 min, to study the kinetics of modification and identify the most reactive lysine residues (17, 18). We found that the lysine residues most amenable to modification could be identified after only a 30 sec-incubation with isoLGs (18). We also studied the effect of CYP27A1 incorporation into phospholipid vesicles prior to isoLG treatment as CYP27A1 is a membrane-bound protein (hence some of its lysine residues may not be available for modification *in vivo*). Indeed, association with liposomes restricted iso[4]LGE₂ modification of CYP27A1 to a smaller set of residues with one of the most reactive residues being then found modified by isoLGs in a biological sample (17). Another finding was that the isoLG-lactam adduct was the most abundant form *in vitro* and the form we observed *in vivo* (17, 18), while the pyrrole and anhydroyrrole adducts were never detected. Finally, by mutating the most reactive residues to arginines, which do not usually interact with isoLGs, we were able to demonstrate that isoLG modification at one specific site could greatly reduce enzyme activity (18). Another application of these data would be the synthesis of a custom peptide corresponding to the sequence of the tryptic or chymotryptic peptides which contained the modified lysine residue. This synthesized peptide could then be modified by stable isotope labeled iso[4]LGE₂, purified, and used as an internal standard for quantifying isoLG modification of a specific protein in biological samples. Alternatively, the peptide could be isotope labeled, e.g. with ¹⁵N, and be modified by unlabeled isoLG.

While this method is focused on a single protein and does not represent a “shotgun” approach, the advantage of our strategy is that it enables the investigation of a low abundance enzyme. In contrast, “shotgun” strategies usually pick up only the most abundant targets, e.g. structural proteins.

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Figure 1.

Isolevuglandins and their adducts with proteins. **(a)** The structures of LGE₂ and **(b)** iso[4]LGE₂. **(c)** IsoLGs react with lysine residues in proteins to form a reversible Schiff base which then rapidly cyclizes to form a stable pyrrole adduct. **(d)** The initial pyrrole adduct may oxidize to isoLG lactams and isoLG hydroxylactams. The adducts may also dehydrate to the anhydro forms. Adapted from (7).

Figure 2. Configuration of the Parameter Editor and Task Editor tabs in Mascot Daemon software for the identification of isoLG-modified lysine residues.

Figure 3.

The MS/MS fragmentation report generated by Mascot consists of several panes of data. (a) The first page of the report displays a listing of search queries with each query line containing information about statistics (e.g. score), peptide sequence and detected modification. Clicking a query number (in underlined blue) loads the corresponding peptide report for that query (b). The peptide report displays the sequence of the identified peptide (in red) in the first line and indicates that it belongs to CYP27A1 in the second line. The next lines display information about the MS/MS scan number examined by the software (highlighted in yellow), the mass of the precursor ion from the full MS scan (highlighted in cyan), and the *m/z* and charge state of the peptide ion (highlighted in light green). The spectrum (red) in the black box is the full MS/MS scan of the fragmented peptide ion, while

the green dashed lines point to the labels indicating the identity of the ions. Below the spectrum are more data regarding the peptide, including any variable modifications detected by the software. The report indicates that an isoLG lactam modification was detected at K11 in the peptide (highlighted in magenta). Beneath this text is a table displaying the m/z of the predicted fragment ions, with the sequence of the peptide displayed (in bold blue) vertically down the center column of the table, and the b-series (peptide fragments numbered from and containing the N-terminus) and y-series (peptide fragments numbered from and containing the C-terminus) ion numbering vertically down the left-hand and right-hand side columns, respectively. Experimentally observed ions matching the predicted m/z are shown in bold red. Identification of both b- and y-series fragment ions which include the modified lysine residue (b11, b13, b14, and y5 – y14), as reflected in their m/z values, and those without it (b2–b4, b7 – b10, and y2 – y4) provides even stronger evidence that the identity of the peptide and location of the isoLG adduct are correct.

Table 1
IsoLGE₂ Adduct Formulas in Mascot

Formulas are as they were entered in Mascot and account for the 2 hydrogen atoms lost from lysine in the reaction with iso[4]LGE₂.

Adduct	Adduct formula	monoisotopic mass (Da)
pyrrole	C ₂₀ H ₂₈ O ₃	316.20
anhydropyrrole	C ₂₀ H ₂₆ O ₂	298.19
lactam	C ₂₀ H ₂₈ O ₄	332.20
anhydrolactam	C ₂₀ H ₂₆ O ₃	314.19
hydroxylactam	C ₂₀ H ₂₈ O ₅	348.19
anhydrohydroxylactam	C ₂₀ H ₂₆ O ₄	330.18
bisanhydrohydroxylactam	C ₂₀ H ₂₄ O ₃	312.17