Chiral lipidomics of monoepoxy and monohydroxy metabolites derived from long-chain polyunsaturated fatty acids

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Abstract A chiral lipidomics approach was established for comprehensive profiling of regio- and stereoisomeric monoepoxy and monohydroxy metabolites of long-chain PUFAs as generated enzymatically by cytochromes P450 (CYPs), lipooxygenases (LOXs), and cyclooxygenases (COXs) and, in part, also unspecific oxidations. The method relies on reversed-phase chiral-LC coupled with ESI/MS/MS. Applications revealed partially opposing enantioselectivities of soluble and microsomal epoxide hydrolases (mEHs). Ablation of the soluble epoxide hydrolase (sEH) gene resulted in specific alterations in the enantiomeric composition of endogenous monoepoxy metabolites. For example, the (R,S) / (S,R)-ratio of circulating 14,15-EET changed from 2.1:1 in WT to 9.7:1 in the sEH-KO mice. Studies with liver microsomes suggested that CYP/mEH interactions play a primary role in determining the enantiomeric composition of monoepoxy metabolites during their generation and release from the ER. Analysis of human plasma showed significant enantiomeric excess with several monoepoxy metabolites. Monoepoxy metabolites were generally present as racemates; however, Ca2+ -ionophore stimulation of whole blood samples resulted in enantioselective formation of monoepoxy metabolites (12S/HETE and 17S-Hydroxydocosahexaenoic acid) and COX-derived metabolites (11R/HETE). Our chiral approach may provide novel opportunities for investigating the role of bioactive lipid mediators that generally exert their physiological functions in a highly regio- and stereospecific manner.

A wide array of bioactive lipid mediators is generated through oxygenation reactions from PUFAs, such as arachidonic acid (AA), EPA, and DHA. Oxygenated PUFAs have also been termed “oxylipins” and comprise metabolites formed by cyclooxygenases (COXs), lipooxygenases (LOXs), and cytochrome P450 (CYP) enzymes as well as nonenzymatic oxidation reactions (1–4). Current methods of targeted lipidomics allow high-throughput, comprehensive, and highly sensitive quantification of oxylipins in biological and clinical samples (5). Most of these analytical approaches rely on LC coupled with MS/MS. Thereby, LC is performed on achiral stationary phases under reversed-phase conditions and MS mostly uses ESI for efficient ionization of the analytes. These advanced methods can measure more than one hundred different oxylipin species in one analytical run, but are unable to distinguish between the enantiomers (5).

The lack of enantiomeric resolution is an inherent property of the achiral-LC-ESI-MS/MS approaches. This feature limits the conclusions that can be drawn regarding the enzymatic versus nonenzymatic origin of oxylipin species or the biological significance of changes in the endogenous oxylipin profile, e.g., in the course of cardiovascular and inflammatory diseases. To address these questions, different strategies of targeted chiral lipidomics are under investigation (6). Chiral-LC has been primarily developed for normal-phase conditions using apolar solvent systems that preclude ESI application for efficient ionization. A way out of this problem is provided by electron capture atmospheric

Supplementary key words tandem mass spectrometry • cytochrome P450 • eicosanoids • lipoxygenase • chiral high-performance liquid chromatography • soluble epoxide hydrolase • microsomal epoxide hydrolase • oxylipins • stereoisomers

Abbreviations: AA, arachidonic acid; CHO, cyclohexene oxide; COX, cyclooxygenase; CYP, cytochrome P450; EDP, epoxydocosapentaenoic acid; EEP, epoxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; FABP, fatty acid binding protein; HDHA, hydroxydocosahexae- noic acid; HEPE, hydroxyeicosapentaenoic acid; IS, internal standard; LLOQ, lower limit of quantitation; LOX, lipooxygenase; mEH, microsomal epoxide hydrolase (EPHIX1); MRM, multiple reaction monitoring; QC, quality control; R2, correlation coefficient; RSD, relative SD; sEH, soluble epoxide hydrolase (EPHX2); S/N, signal-to-noise; SPE, solid phase extraction; TTPU, N-[1-(1-oxopropyl)-4-piperidinyl]N-[4-(trifluoromethoxy)phenyl]urea.

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pressure chemical ionization (ECAPCI)-MS. This method can be coupled with normal-phase chiral-LC and requires prior derivatization of oxylipins, e.g., into their pentafluorobenzyl esters (6). Using this sensitive approach, a recent study showed stereoselective responses to in vitro stimulation of human blood samples regarding the formation of HETEs (7). An alternative strategy takes advantage of chiral stationary phases that are compatible with water-containing polar mobile phases. This approach allows performing reversed-phase chiral-LC coupled with ESI-MS/MS detection and quantitation of the resolved stereoisomers. Reversed-phase chiral-LC-ESI-MS/MS has been successfully used for the determination of enantiomers as well as diastereomeric epimers of pro-resolving lipid mediators (8–10).

The present study was aimed at developing an analytical system suitable for comprehensive profiling of regio- and enantiomeric monoepoxy and monohydroxy metabolites in biological samples. The metabolites to be targeted included epoxyicosatetraenoic acids (EETs) and HETEs derived from AA, epoxyeicosatetraenoic acids (EEQs) and hydroxyeicosapentaenoic acids (HEPEs) derived from EPA, and epoxydocosapentaenoic acids (EDPs) and hydroxydocosahexaenoic acids (HDHAs) derived from DHA. While each of the monoepoxy metabolite regioisomers can be formed as (R,S)- or (S,R)-enantiomers, the hydroxyl group in the various regioisomeric monohydroxy metabolites can exist in R- or S-configuration. After optimizing the set-up and conditions for reversed-phase chiral-LC-ESI-MS/MS, we tested the applicability of the established procedure first by analyzing the enantiomeric mixtures of soluble epoxide hydrolases (sEHs) and microsomal epoxide hydrolases (mEHs) in metabolizing CYP-derived EETs, EEQs, and EDPs. Subsequent studies served to elucidate the profile of the targeted oxylipin stereoisomers in plasma and liver samples from WT and sEH-KO mice, as well as in human plasma.

**MATERIALS AND METHODS**

**Chemicals and reagents**

The enantiomers of 8,9-, 11,12-, and 14,15-EET (11), as well as of 17,18-EEQ and 19,20-EDP (12) were prepared as described previously by resolving the racemic mixtures (±) of the respective monoepoxides, as purchased from Cayman Chemicals, using chiral-LC on Chiralcel OD or OB columns (Daicel, Illkirch, France). Authentic samples of 17(R),18(S)- and 17(S),18(R)-EEQ were produced using recombinant CYP1A1 and CYP1B2, respectively (13). It was assumed that the 19,20-EDP enantiomers elute in the same order as the 17,18-EEQ enantiomers; however, authentic standards were not available to verify their identities. The (R,S) and (S,R) enantiomers collected from chiral-LC were quantitated using achiral-LC-MS/MS as described below. All racemic oxylipins as well as the currently available set of R- and S-enantiomers of HETEs, HEPEs, and HDHAs were purchased from Cayman Chemicals. If authentic stereoisomers were not available for a given oxylipin, steric configurations were not assigned; instead, they were designated as peak 1 (P1) and P2, according to the order of their elution from chiral-LC. The internal standard (IS) solution was prepared in acetonitrile and contained 0.5 μg/ml each of 15(S)-HETE-d8 and ±8,9-EET-d11.

Bond Elut Certify II columns (3 ml, 200 mg sorbent) for solid phase extraction (SPE) were purchased from Agilent Technologies. Acetonitrile, methanol, glacial acetic acid, water (LC-MS grade), and buffers were purchased from VWR International. N-[1-(1-oxopropyl)-4-piperidinyl]N-[4-(trifluoromethoxy)phenyl]urea (TPPU) was bought from Cayman Chemicals. Cyclohexene oxide (CHO), BSA (fatty acid free), and ketamine/xyazine K-113 were purchased from Sigma-Aldrich and Na-heparin from Braun Melsungen.

**LC-MS/MS analysis**

Columns containing chiral polysaccharide-based stationary phases were purchased from Phenomenex (Aschaffenburg, Germany). For the “chiral-1” method (compare Fig. 1), Lux-Celulose-3 (150 × 2 mm, 3 μm particles) was coupled upstream with an Agilent ZORBAX Eclipse Plus C18 column (50 × 2.1 mm, 1.8 μm particles). Mobile phase was a linear gradient of methanol/water/glacial acetic acid from 70:30:0.05 to 80:20:0.05 (v/v/v) over 16 min at a flow rate of 0.4 ml/min, followed by washing with 100% methanol over 4 min. For the “chiral-2” method, Lux-Amylose-1 (150 × 2 mm, 3 μm particles) was coupled upstream with an Agilent ZORBAX SB-C8 column (50 × 2.1 mm, 1.8 μm particles). Mobile phase was a linear gradient of acetonitrile/methanol/water/glacial acetic acid from 27:37:50:0.05 to 63:37:20:0.05 (v/v/v/v) over 30 min at a flow rate of 0.4 ml/min. Before use, the chiral column systems were carefully adjusted to appropriate pressure (pressure maximum set at 300 bar) by increasing the flow rate over 10 min from 0 to 0.4 ml/min, followed by an equilibration period of 1 h. Retention times of analytes became stable after two runs, as tested using a mix of authentic standard compounds for quality control (QC). Routinely, an equilibration time of 15 min between successive analytical runs was used. Before long-term storage, the chiral columns were washed with acid-free 95% organic mobile phase.

For the achiral system, an Agilent Zorbax Eclipse Plus C18 column (150 × 2.1 mm, 1.8 μm particles) was used as stationary phase. Chromatography was done under gradient conditions at a flow rate of 0.3 ml/min starting with acetonitrile/water/acetate acid in a ratio of 5:95:0.05 (v/v/v) for 0.5 min, followed by increases to 56:44:0.05 within 5 min, to 61:39:0.05 within 5 min, and finally to 87:13:0.05 within 13 min, followed by washing the column with 98:2:0.05 for 6.5 min.

All columns were kept at 40°C and the autosampler at 6°C. The injection volume was 10 μl (15 μl for achiral analysis). LC-MS/MS analysis was performed using an Agilent 6490 Triple Quad mass spectrometer (Agilent Technologies, Santa Clara, CA) coupled with an UHPLC system of the Agilent 1290 Infinity series. ESI operated in the negative ionization mode. The instrument parameters and conditions for multiple reaction monitoring (MRM) are given in supplemental Table S1. Peak detection, integration, and quantitation were done using Agilent MassHunter software.

**Calibration and sensitivity**

15(S)-HETE-d8 and ±8,9-EET-d11 served as ISs for quantifying monohydroxides and monoepoxides, respectively. Ten-point-calibration curves were obtained based on analyte/IS peak area ratios after injecting 10 μl of stock solutions in acetonitrile that contained 0–25 ng of the analytes and 5 ng IS per 100 μl. Limit of detection was defined as the lowest concentration that gave a signal-to-noise (S/N) ratio ≥3. Lower limit of quantitation (LLOQ) was defined as the lowest concentration that gave a S/N ratio ≥10. The S/N ratio was calculated using the adjacent area corresponding to one peak width of the analyte as reference.
Accuracy and precision

Accuracy and precision were tested analyzing QC samples. The QC samples were used at concentrations corresponding to the low, mid, and upper parts of the linear calibration curves and were measured three times. “Accuracy I” represents intra-day deviations and was expressed as percent difference of the measured mean from the expected concentration as calculated from the respective calibration function. Precision was defined as the relative SD (RSD) of the three repeated measures. “Accuracy II” of the chiral methods was expressed as percent difference between the mean sum measured for a given pair of enantiomers and the corresponding total metabolite level as measured by the achiral method.

Matrix effects

Deuterated standards were purchased from Cayman Chemicals [±8,9-EET-d11, ±11,12-EET-d11, ±14,15-EET-d11, 5(S)-HETE-d8, 12(S)-HETE-d8, and 15(S)-HETE-d8]. A mix of these standards (5 ng each) was used to spike extracts (n = 6 per group) prepared as described below from 30 mg murine liver tissue or 0.5 ml human plasma reconstituted in 100 µl of acetonitrile/water (60:40). Identically spiked solvent samples served as control. Matrix effects were calculated as percentage of the peak areas measured by chiral-LC-MS/MS after adding the standards to the extracts compared with the pure solvent.

Determination of epoxide hydrolase activities

Recombinant human sEH was purchased from Cayman Chemicals and diluted in incubation buffer containing 0.1% fatty acid-free BSA. Pooled human liver microsomes were purchased from Thermo Fisher Scientific. Murine liver microsomal and cytosolic fractions were prepared as described previously (14). The hydrolase assays were performed at 30°C in 300 µl of Tris-HCl buffer (50 mM) with 0.01% fatty acid-free BSA, pH 7.5. Substrates were used at a final concentration of 10 µM. To determine sEH-mediated hydrolyses, the assays were started by adding the indicated amounts of human sEH or liver cytosolic fraction prepared from WT mice. mEH activities were determined using human liver or murine liver microsomal protein (prepared from sEH-KO mice). Incubations with human liver microsomes were performed in the presence of 2 µM TPPU. The reactions were stopped by adding 15 µl of 400 mM citric acid and 1 ml of ethyl acetate. The ethyl acetate extracts were then evaporated, dissolved in acetonitrile/water (60:40), and analyzed using chiral-LC-MS/MS.

CYP-mEH interactions in liver microsomes

Incubations for analyzing liver microsomal AA metabolism were performed at 37°C in a total volume of 100 µl of 100 mM potassium phosphate buffer (pH 7.2) containing 80 µg of the microsomal protein and the substrate at a final concentration of 10 µM. After preincubating the microsomes with AA for 5 min, reactions were started with NADPH (1 mM final concentration) and terminated after 10 min by adding 5 µl of 0.4 M citric acid. In control experiments, NADPH was omitted. To exclude potentially contaminating sEH activities, murine liver microsomes were prepared from sEH-KO mice and the reactions with human liver microsomes were performed in the presence of the sEH inhibitor, TPPU (2 µM). CHO was used to inhibit mEH activities (15, 16); the final CHO concentrations were 250 µM with murine or 800 µM with human liver microsomes. Incubations containing 0.1% ethanol were performed as vehicle controls. Reaction products were extracted into ethyl acetate, evaporated under nitrogen, dissolved in acetonitrile/water (60:40), and analyzed using chiral-LC-MS/MS.

Extraction of oxylipins from biological samples

Plasma samples (0.3–0.5 ml) or 10–20 mg aliquots of homogenized liver tissue mixed with 0.5 ml of distilled water containing 4 µM TPPU, were filled up with 1.5 ml of acetonitrile containing 50 µg of butylated hydroxytoluene and the IS [5 ng each of 15(S)-HETE-d8 and ±8,9-EET-d11]. Next, 0.5 ml of 10 M sodium hydroxide were added and the samples were incubated for 30 min at 60°C. After alkaline hydrolysis, 0.5 ml of acetic acid/water (38:42, v/v) and 3.5 ml of phosphate buffer (Sorensen’s buffer, pH 6.0, containing 5% methanol) were added. The pH was controlled and, if necessary, adjusted to pH 6.0. The samples were then cleared by centrifugation and extracted by SPE. The SPE columns were preconditioned with 3 ml of methanol, followed by 6 ml of Sorensen’s buffer, pH 6.0, containing 5% methanol. Columns were loaded with the samples and washed with 3 ml of methanol/water (1:1, v/v). Metabolites and IS were eluted with 2 ml of hexane/ethyl acetate (75:25, v/v) containing 1% acetic acid. The eluates were transferred into vials preloaded with 20 µl of glycerol/methanol (1:9, v/v) and then evaporated to dryness on a heating block at 40°C under a stream of nitrogen. Residues were dissolved in 100 µl of acetonitrile/water (60:40) and either stored at −80°C or immediately applied to LC-MS/MS.

Animal experiments

All animal experiments were performed in accordance with the guidelines of the Charité University Berlin and approved by local German authorities (LaGeSo, G0146/16, Berlin, Germany). WT and sEH-KO mice were originally established by Boehringer-Ingelheim Pharmaceuticals and were then further backcrossed for nine generations onto C57BL/6J before being used in our studies (17, 18). The animals were kept under specific pathogen-free conditions with a standard 12:12 h light-dark cycle and had ad libitum access to water and standard chow. Starting with an age of 10–13 weeks, male WT and sEH-KO mice (n = 6 per group) received a diet enriched in EPA and DHA over a period of 3 weeks. The diet (EF R/M, containing 5% sunflower and 2.5% Omacor oil from SNIFF Spezialitäten GmbH, Soest, Germany) was composed as described in detail previously (12). Mice were anesthetized with ketamine/xylazine (240 mg/kg) containing heparin (220 IU/kg). Blood (0.5–1.0 ml) was collected and plasma was prepared immediately by centrifugation (10,000 g, 10 min). Plasma as well as harvested liver and other organ samples were snap-frozen in liquid nitrogen and stored at −80°C. Before analysis, organs were homogenized in liquid nitrogen using a BioPulverizer (BioSpec Products Inc., USA).

Human blood

The human blood samples analyzed in the present study originated from a previous trial, where we treated 20 healthy volunteers with an EPA/DHA supplement and analyzed concomitant changes in the formation of AA, EPA, and DHA-derived metabolites (19). The blood samples used here for chiral-LC-MS/MS analysis were withdrawn after the participants received dietary EPA/DHA supplementation for 8 weeks. One set of the corresponding plasma samples (n = 6; three male and three female) was immediately prepared by centrifugation. Another set of plasma samples was prepared after in vitro incubation of fresh whole blood samples (4.5 ml) with the Ca2+-ionophore, A23187 (50 µM), or its vehicle (1% DMSO) for 30 min at 37°C (n = 6 per group). Samples were stored at −80°C until analysis.

Statistics

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Inc., La Jolla, CA). All tests were done with the nonparametric Mann-Whitney U test. A probability value of
RESULTS

Set-up of the analytical system

Figure 1 shows the principal setup of the analytical procedure developed for comprehensive profiling of regio- and stereoisomeric monoeoxy and monohydroxy metabolites in biological samples. Using aliquots of the extracted metabolites, chiral-LC was performed on two different stationary phases, each coupled with ESI-MS/MS detection. The chiral-1 and chiral-2 methods resolved and enabled measurement of distinct subsets of enantiomeric metabolites, comprising most of the targeted monoeoxides on Cellulose-3 (Table 1) and all monohydroxides as well as several mid-chain monoeoxides on Amylose-1 (Table 2), respectively. A third aliquot was used to perform reversed-phase LC on a C18 column followed by ESI-MS/MS detection. This “achiral” method was included to obtain reference data on the total levels of all targeted metabolites, independent of their enantiomeric composition.

The polysaccharide-based chiral phases selected (Cellulose-3 and Amylose-1) are compatible with polar solvent systems, such as methanol/water or acetonitrile/water containing 0.05% acetic acid. This property made it possible to perform chiral-LC under reversed-phase conditions and to use ESI-MS/MS in the negative mode for highly sensitive detection of the resolved stereoisomers. The conditions and parameters for MRM were identical for chiral- and achiral-LC (supplemental Table S1). An initially occurring problem was the insufficient resolution of some enantiomers that display identical precursor ion to diagnostic fragment transitions, as exemplified by the enantiomers of 15-HETE and 14,15-EET (compare supplemental Table S1 and supplemental Fig. S1). The problem was solved by coupling the chiral columns upstream with a short achiral C18 or C8-column, that preseparated the monohydroxy and epoxy metabolites derived from the same parental PUFA (supplemental Fig. S1).

Features and validation of the analytical system

Chiral-LC-ESI-MS/MS was established and validated using authentic standard compounds that comprised 12 monoeoxides and 12 monohydroxides metabolites derived from AA, EPA, and DHA (supplemental Table S2). As summarized in Tables 1 and 2, baseline-resolution of the corresponding enantiomers, as defined by $R \geq 1.5$ (20), was achieved with most of the metabolites using either Cellulose-3 or Amylose-1 as stationary phase. Exceptions were the enantiomers of 19-HETE, 11,12-EEQ, and 13,14-EDP that were not resolved at all on Cellulose-3 and only moderately ($R$ = 0.6) on Amylose-1. Both the extent of chiral resolutions and the individual retention times of the enantiomers were highly reproducible under the reversed-phase conditions used (Tables 1, 2). Linear calibration curves with correlation coefficients ($R^2$) higher than 0.99 were obtained for the quantitation of individual metabolites based on the changes in the relative peak area in response to different target compound/IS-concentration ratios. LLOQs were specific for each of the enantiomers and ranged from <0.05 to 0.25 ng absolute, corresponding to 5–25 pg on column (Tables 1, 2).

Intraday precision and accuracy were determined using QC samples that contained a mixture of all analytes in three different concentrations (supplemental Table S2). Precision was satisfactory with all targeted metabolites, as reflected by RSD $\leq 15\%$ (supplemental Table S3). Accuracy ranged, in general, from 1% to 20% using QC1 [exceptions: 9(S)-HETE and 19(R)-HETE with about 23%] and was better than 15% for most analytes in QC2 [exceptions: 9(S)-HETE and 17(S)-HDHA with about 16%]; see Accuracy I in supplemental Table S3. For the highest concentration tested (QC3), accuracy was not satisfactory (deviations between 15% and 27.5%) for several monohydroxy metabolites, including, in particular, those present in concentrations already reaching the upper limit of their linear calibration curves.
included keeping the column temperature constant at 40°C to maintain a high degree of reproducibility. Critical factors included the chiral methods described above, experiments (see supplemental Table S3) and quantitation in biological matrices were still possible.

| Compound | LLOQ (ng) | Linear Range (ng) | \( R^2 \) | Retention Time (RSD) | Resolution \( R' \) (RSD) |
|----------|-----------|-------------------|---------|----------------------|-------------------------|
| 8(R),9(S)-ETE | 0.25 | 0.25–25 | 0.9988 | 13.02 (0.3) | 2.0 (5.4) |
| 8(S),9(R)-ETE | 0.25 | 0.25–25 | 0.9989 | 13.93 (0.2) | 1.6 (5.7) |
| 14(R),15(S)-ETE | 0.10 | 0.1–25 | 0.9985 | 12.98 (0.2) | 2.5 (9.4) |
| 14(S),15(R)-ETE | 0.10 | 0.1–25 | 0.9984 | 13.68 (0.2) | |
| 8,9-EEQ peak 1 | 0.10 | 0.05–25 | 0.9994 | 12.23 (0.3) | 2.2 (2.9) |
| 8,9-EEQ peak 2 | 0.10 | 0.05–25 | 0.9975 | 13.25 (0.4) | 1.5 (4.3) |
| 14,15-EEQ peak 1 | <0.05 | 0.05–25 | 0.9986 | 11.54 (0.3) | |
| 14,15-EEQ peak 2 | <0.05 | 0.05–25 | 0.9777 | 12.41 (0.2) | |
| 17(R),18(S)-EEQ | <0.05 | 0.1–25 | 0.9886 | 11.20 (0.2) | 2.7 (4.9) |
| 17(S),18(R)-EEQ | <0.05 | 0.1–25 | 0.9865 | 11.78 (0.2) | |
| 16,17-EDP peak 1 | 0.25 | 0.1–25 | 0.9886 | 14.09 (0.2) | 2.2 (5.4) |
| 16,17-EDP peak 2 | 0.25 | 0.1–25 | 0.9977 | 15.89 (0.2) | |
| 19(R),20(S)-EDP | 0.25 | 0.25–25 | 0.9989 | 14.68 (0.3) | |
| 19(S),20(R)-EDP | 0.25 | 0.25–25 | 0.9991 | 15.65 (0.3) | |

* Nanograms absolute.

** RSD (%) related to nine QC samples at three different concentrations.

\( R = 1.18 \cdot \frac{(t_{R} - t_{0})}{(FWHM + FWMH)} \) where \( t_{R} \) is retention time (minutes) and FWHM is full peak width at half maximum (minutes); formula according to (20).

Technical notes and conditions for the analysis of biological samples

While establishing the chiral methods described above, we observed that special care is needed to achieve and maintain a high degree of reproducibility. Critical factors included keeping the column temperature constant at 40°C and avoiding pressure maxima above 300 bar, as well as rapid pressure changes. Before daily use, the columns had to be carefully equilibrated with the desired solvent system and checked for performance and accuracy of quantitation using a mix of authentic standard compounds. Moreover, at a flow rate of 0.4 ml/min, an equilibration time of

| TABLE 1. Chromatographic parameters of the chiral-1 method |
|-----------------------------|-----------------------------|-----------------------------|
| Compound | LLOQ (ng) | Linear Range (ng) | \( R^2 \) | Retention Time (RSD) | Resolution \( R' \) (RSD) |
| 5(R)-HETE | <0.05 | 0.05–10 | 0.9999 | 22.05 (0.0) | 5.7 (6.6) |
| 5(S)-HETE | <0.05 | 0.9872 | 23.47 (0.0) | 3.6 (10.7) |
| 9(R)-HETE | 0.10 | 0.25–25 | 0.9993 | 21.79 (0.1) | 6.4 (4.7) |
| 9(S)-HETE | 0.10 | 0.9944 | 20.03 (0.1) | 4.4 (4.0) |
| 11(R)-HETE | <0.05 | 0.25–10 | 0.9974 | 21.51 (0.1) | 5.4 (3.3) |
| 11(S)-HETE | <0.05 | 0.9995 | 23.02 (0.1) | 3.9 (4.6) |
| 12(R)-HETE | <0.05 | 0.05–10 | 0.9871 | 22.87 (0.0) | 8.8 (5.3) |
| 12(S)-HETE | <0.05 | 0.9911 | 24.45 (0.1) | 6.4 (18.5) |
| 15(R)-HETE | 0.25 | 0.05–10 | 0.9872 | 22.02 (0.1) | 4.4 (4.0) |
| 15(S)-HETE | 0.25 | 0.9909 | 24.54 (0.1) | 3.9 (4.6) |
| 19(R)-HETE | 0.25 | 0.25–10 | 0.9986 | 19.57 (0.1) | 4.4 (4.0) |
| 19(S)-HETE | 0.25 | 0.9899 | 19.41 (0.1) | 3.9 (4.6) |
| 5(R)-HEPE | <0.05 | 0.05–10 | 0.997 | 19.51 (0.1) | 3.9 (4.6) |
| 5(S)-HEPE | <0.05 | 0.9907 | 20.59 (0.1) | 4.4 (4.0) |
| 9(R)-HEPE | <0.05 | 0.05–10 | 0.9933 | 19.19 (0.0) | 4.4 (4.0) |
| 9(S)-HEPE | <0.05 | 0.9939 | 20.11 (0.0) | 5.7 (14.8) |
| 12(R)-HEPE | 0.25 | 0.25–25 | 0.9931 | 20.18 (0.1) | 4.4 (4.0) |
| 12(S)-HEPE | 0.10 | 0.9893 | 21.61 (0.1) | 4.9 (5.1) |
| 15(R)-HEPE | <0.05 | 0.05–10 | 0.9929 | 19.38 (0.0) | 4.9 (5.1) |
| 15(S)-HEPE | <0.05 | 0.9916 | 20.60 (0.1) | 4.9 (5.1) |
| 18-HEPE peak 1 | <0.05 | 0.05–10 | 0.9925 | 18.45 (0.1) | 3.0 (2.2) |
| 18-HEPE peak 2 | <0.05 | 0.9929 | 19.14 (0.1) | 3.0 (2.2) |
| 17(R)-HDHA | <0.05 | 0.25–10 | 0.9993 | 21.85 (0.1) | 4.9 (9.1) |
| 17(S)-HDHA | <0.05 | 0.9938 | 22.97 (0.1) | 4.9 (9.1) |
| 11(R),12(S)-EET | <0.05 | 0.5–10 | 0.9979 | 26.77 (0.0) | 1.5 (7.7) |
| 11(S),12(R)-EET | <0.05 | 0.994 | 26.37 (0.1) | 1.5 (7.7) |
| 11,12-EEQ peak 1 | <0.05 | 0.25–25 | 0.9994 | 23.55 (0.0) | 0.7 (10.1) |
| 11,12-EEQ peak 2 | <0.05 | 0.9992 | 23.76 (0.1) | 0.7 (10.1) |
| 7,8-EDP peak 1 | <0.05 | 0.25–10 | 0.9975 | 26.18 (0.0) | 3.6 (5.0) |
| 7,8-EDP peak 2 | <0.05 | 0.997 | 27.18 (0.1) | 3.6 (5.0) |
| 13,14-EDP peak 1 | <0.05 | 0.25–10 | 0.9979 | 25.98 (0.0) | 0.7 (9.6) |
| 13,14-EDP peak 2 | <0.05 | 0.9984 | 26.20 (0.1) | 0.7 (9.6) |

* Nanograms absolute.

** RSD (%) related to nine QC samples at three different concentrations.

\( R = 1.18 \cdot \frac{(t_{R} - t_{0})}{(FWHM + FWMH)} \) where \( t_{R} \) is retention time (minutes) and FWHM is full peak width at half maximum (minutes); formula according to (20).

* Baseline separation of the corresponding enantiomers was not reached (\( R < 1.5 \)); however, reliable validation experiments (see supplemental Table S3) and quantitation in biological matrices were still possible.

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15 min between successive analytical runs was important for assuring constant retention times and resolution of the enantiomers.

As outlined in detail below, the established procedure was applied to measure the levels of enantiomeric monoepoxy and monohydroxy metabolites in biological samples. Figure 2 shows several representative examples of chromatograms as obtained during the chiral analyses of murine liver and plasma samples. Quantitation relied on adding known amounts of 15(S)-HETE-d8 and ±8,9-EET-d11 as ISs. After alkaline hydrolysis and solid-phase extraction, the overall recovery of the ISs ranged between 50% and 80% (data not shown). Potential matrix effects were evaluated using a mixture of commercially available deuterated monoepoxy and monohydroxy metabolites of AA. Indicating the absence of major matrix effects, recovery of the deuterated compounds spiked after solid-phase extraction ranged between 90% and 115%, as measured by both the chiral and achiral methods (supplemental Table S4).

Another criterion for evaluating the performance of the chiral methods was the concordance of the sum of each pair of measured enantiomers with the total level of the respective regioisomer as determined by the achiral reference method. Using the QC-mix of authentic standard compounds, the deviations were less than 20% for all concentrations tested, except for 13,14-EDP (21.5%) and 11,12-EEQ (20.2%) with QC1 (see Accuracy II in supplemental Table S3). Essentially concordant total metabolite levels were also obtained with biological samples, if analyzed in parallel by the chiral and achiral methods (supplemental Tables S5, S6).

**Stereosepecificities of soluble and mEHs**

To test the applicability of the novel chiral methods, we first analyzed the regio- and stereosepecificity of sEH, an enzyme whose enantioselectivity in EET hydrolysis had already been known from previous studies (21). Murine sEH, as present in the cytosolic fraction of liver homogenates, displayed a pronounced enantioselectivity in favor of hydrolyzing the \( R,S \)-enantiomers of 14,15-EET, 17,18-EEQ, and 19,20-EDP. In this experiment, the liver cytosolic fraction was added to an equimolar mixture (10 \( \mu \)M each) of all three racemic monoepoxides (Fig. 3).

Subsequent experiments were performed to recognize potential differences between sEH and mEH regarding their enantioselectivities. To characterize mEH activities, we used liver microsomes isolated from sEH-KO mice and human liver microsomes in the presence of a potent sEH inhibitor (TPPU, 2 \( \mu \)M). Murine liver cytosol and purified recombinant human sEH served for direct comparison. Enantioselectivities were characterized by following the time course by which the \((R,S)\)- and \((S,R)\)-enantiomers were enzymatically hydrolyzed and thus disappeared from the originally racemic substrate. Using this method, essentially opposing enantioselectivities became apparent comparing the sEH and mEH enzymes (Figs. 4, 5). Taking 14,15-EET as an example, murine and human sEH preferentially hydrolyzed the \((R,S)\)-enantiomer, whereas the microsomal enzymes started to hydrolyze the \((R,S)\)-enantiomer only after almost completely metabolizing the corresponding \((S,R)\)-enantiomer. Clear differences between sEH and mEH enantioselectivities were also found using \(\pm 11,12\)-EET and \(\pm 17,18\)-EEQ (Figs. 4, 5) or \(\pm 8,9\)-EET and \(\pm 19,20\)-EDP as substrates (supplemental Fig. S2).

**Fig. 2.** Representative chiral-LC-ESI-MS/MS chromatograms. Shown are examples for the enantiomeric resolution of monoepoxy and monohydroxy metabolites extracted from WT murine liver and plasma samples. Analysis was performed using the chiral-1 or chiral-2 method as appropriate for the different analytes (see Tables 1, 2). Labeled are the \((R)\)/(\(R,S\))-enantiomer (I) and the \((S)\)/(\(S,R\))-enantiomer (II).
CYP-mEH interaction and the stereospecificity of microsomal monoepoxide formation

Specific mEH activities are by orders of magnitude lower than those of sEH enzymes, if determined with exogenously added substrates. However, a close physical association of microsomal CYP and mEH enzymes has been recently discovered that may largely facilitate the access to endogenously generated monoepoxides (22, 23). Accordingly, we hypothesized that CYP-mEH interactions might also modulate the stereospecificity of microsomal metabolite formation. To test this hypothesis, we analyzed the NADPH-dependent generation of AA-derived monoepoxides (EETs) by murine and human liver microsomes in the absence and presence of the mEH inhibitor, CHO (15, 16). CHO was used in concentrations sufficient to inhibit microsomal DHET formation by more than 90% (data from achiral analysis, not shown). Potentially contaminating sEH activities were excluded by using liver microsomes from sEH-KO mice or inhibiting the enzyme with TPPU. As shown in Fig. 6, the liver microsomes generated EETs as well as 19-HETE as major primary metabolites. CHO-mediated mEH inhibition was accompanied with significant changes in the enantiomeric composition of the monoepoxides. With murine liver microsomes, mEH inhibition increased the 11(\(R\)),12(\(S\))-EET level about 4-fold compared with the vehicle control (Fig. 6A). With human liver microsomes, the most pronounced effect of mEH inhibition was an almost 5-fold increase of 14(\(S\)),15(\(R\))-EET (Fig. 6B). The specific enhancements observed were in line with the enantioslectivity of the mEH enzymes as revealed before with exogenously added monoepoxides (compare Figs. 4 and 5). These data suggest that mEH enzymes rapidly hydrolyze distinct monoepoxide enantiomers following their formation by microsomal CYP enzymes. In contrast, mEH inhibition did not affect the enantiomeric composition of 19-HETE, a major monohydroxy metabolite of AA, that was simultaneously produced by liver microsomal CYP enzymes (Fig. 6).

In vivo formation of enantiomeric monoepoxides in WT and sEH-KO mice

Ablation of the sEH gene (EPHX2) has been widely used to investigate the protective role of PUFA monoepoxides in cardiovascular and inflammatory diseases (24, 25); however, the impact of sEH deficiency on the enantiomeric composition of endogenous monoepoxy metabolites remained unclear. To address this question, we analyzed liver and plasma samples from WT and sEH-KO mice. The animals were fed with a diet enriched in EPA and DHA to cover the whole range of biologically active metabolites targeted by our chiral method. Compared with WT, sEH-KO mice showed significantly increased hepatic and plasma levels of the (\(R\),\(S\))-enantiomers of 14,15-EET, 17,18-EEQ, and 19,20-EDP, whereas the corresponding (\(S\),\(R\))-enantiomers were only slightly increased or not affected at all (Fig. 7). Similar changes of the enantiomeric ratio were observed for other monoepoxides, except the midchain-epoxides, 11,12-EET, 11,12-EEQ, and 13,14-EDP, that were not affected by sEH-KO (supplemental Tables S5, S6). In contrast, neither the total levels...
nor the enantiomeric compositions were different with several other monoepoxides comparing liver (supplemental Table S5) or plasma samples (supplemental Table S6) from WT and sEH-KO mice. It is noteworthy that the specific increases observed in sEH-KO mice for the (R,S)-enantiomers of 14,15-EET, 17,18-EEQ, and 19,20-EDP as well as for 8(S),9(R)-EET were in line with the preferential hydrolysis of these enantiomers by the murine sEH enzyme under in vitro conditions (compare Figs. 3–5 and supplemental Fig. S2).

Chiral profiling of oxylipins in human plasma

As a further example of potential applications, we used the novel chiral methods for determining the enantiomeric composition of monoepoxy and monohydroxy metabolites in human plasma. The samples were taken from healthy volunteers (n = 6) recruited in a previous study after the subjects received dietary EPA/DHA supplementation for 8 weeks (19). All targeted metabolites, except 8,9-EEQ, were present above their LLOQ levels and could be quantitated. Among the monoepoxy metabolites analyzed, a significant enantiomeric excess was detected with 8,9-EET, 11,12-EEQ, 17,18-EEQ, and 19,20-EDP as well as for 8(S),9(R)-EET were in line with the preferential hydrolysis of these enantiomers by the murine sEH enzyme under in vitro conditions (compare Figs. 3–5 and supplemental Fig. S2).

stimulating monohydroxy metabolite de novo synthesis by treating whole blood samples with the Ca²⁺-ionophore, A23187. In these experiments, we again used blood samples from healthy volunteers 8 weeks after dietary EPA/DHA supplementation (n = 6 per group). As shown in Fig. 9, Ca²⁺-ionophore stimulation resulted in markedly increased levels of the 12(S)-HETE, 12(S)-HEPE, and 17(S)-HDHA, in line with the notion that these metabolites were produced by the highly stereospecific action of LOX enzymes. It is noteworthy that 18-HEPE, the precursor of resolvins, was also increasingly generated in an enantiospecific manner upon Ca²⁺-ionophore stimulation (Fig. 9). Furthermore, 11(R)-HETE, a putative COX-derived metabolite, was specifically increased, whereas neither the formation nor the enantiomeric composition of 9-HETE was affected (Fig. 9).

DISCUSSION

In the present study, we established an analytical system suitable for comprehensive profiling of regio- and stereoisomeric oxylipins in biological samples. The targeted metabolites comprised monoepoxy and monohydroxy metabolites as specifically generated from AA, EPA, and DHA through CYP-, LOX-, and COX-catalyzed reactions as well as, in part, unspecific free radical-mediated oxygenation.
reactions. The resolution and quantitation of the corresponding enantiomers relied on reversed-phase chiral-LC coupled with highly sensitive ESI-MS/MS detection. As exemplified by our studies on the role of epoxide hydrolases in determining the enantiomeric composition of endogenous monoepoxides, this approach may provide novel opportunities for investigating the formation, degradation, and action of CYP epoxygenase-derived bioactive lipid mediators. Moreover, the approach also has potential for distinguishing between monohydroxy metabolites formed enantioselectively by LOX or COX enzymes and those generated as racemates via nonenzymatic oxidation reaction.

In our hands, the chiral- and achiral-LC-ESI-MS/MS methods reached almost equally high sensitivities when performed in direct comparison and using the same instrumentation as well as parameters and conditions for MRM-based quantitation of the metabolites. Nonetheless, our and other strategies to couple chiral-LC with MS/MS detection (6–10) are rather at the beginning, if compared with the advanced achiral methods that allow high-throughput applications and the simultaneous measurement of more than one hundred different oxylipins (5). The number of oxylipin species targeted by chiral methods can certainly be largely increased, e.g., by including the metabolites of C18-PUFAs. However, none of the currently available chiral stationary phases seem to have the property of resolving the complete set of regio- and stereoisomeric oxylipins, as ideally needed for screening assays. We had to perform analytical runs on two different chiral phases in order to resolve distinct subsets, comprising most of the targeted monoepoxides on Lux Cellulose-3 and all monohydroxides as well as several mid-chain monoepoxides on Lux Amylose-1. Other investigators used Chiralpak AD-RH, Lux Amylose-2, or Lux Amylose-1 with 250 × 4.6 mm size to run chiral-LC coupled with ESI-MS/MS for the targeted lipidomics of resolvins and other specialized pro-resolving lipid mediators (8–10). Relatively long equilibration times needed before and in between successive analytical runs (see Technical notes and conditions for the analysis of biological samples in the Results section) are another problem concerning the moderate throughput in our chiral approach. Future development of chiral phases may help to overcome these problems, e.g., by improving pressure tolerance, enlarged column length, using smaller particle sizes, and/or novel chiral ligands.

Stereoselectivity had been observed already in the early stages of research that established CYP-dependent eicosanoid formation as the third branch of the AA cascade (26, 27). Enantioselectivities were discovered analyzing CYP-dependent EET formation (28–31), sEH-mediated EET hydrolysis (21), and the incorporation of EETs into membrane phospholipids (32). CYP enzymes were also shown to catalyze the epoxidation of EPA and DHA to EEQs and EDPs in a regio- and stereospecific manner (13,
Importantly, it became apparent that EET-, EEQ-, and EDP-induced biological effects are stereospecific, whenever this question was explicitly addressed. Examples include enantiospecific vasodilatory responses (35, 36), effects on ion channels (37), and Ca²⁺ handling (12) in cardiomyocytes, as well as the induction of cAMP formation and signaling in endothelial cells (38). Our findings with liver microsomes suggest that CYP-mEH interactions may play a primary role in determining the enantiomeric composition of monoepoxy metabolites that are generated in and released from the ER, before they can exert their autocrine and paracrine actions. Obviously, CYP-mEH complex formation does not only facilitate the rapid hydrolysis of certain EET, EEQ, and EDP regioisomers as discovered recently (23), but simultaneously modulates their enantioselectivity. We also found that sEH and mEH enzymes display different and, in part, even opposing enantioselectivities. Combined with the stereospecificities of individual CYP enzymes, this state-of-affairs creates a high degree of complexity regarding the factors that potentially determine the endogenous profile of regio- and stereoisomeric fatty acid epoxides. The enantioselectivity of murine sEH was reflected by specific increases of the (R,S)-enantiomers of 14,15-EET, 17,18-EEQ, and 19,20-EDP in liver and plasma of sEH-KO compared with WT mice. Moreover, we observed that the fold-change was much more pronounced with the enantiomers than with the total levels of the corresponding regioisomers. This shift in enantiomeric composition has not been analyzed before, but might have significantly contributed to the beneficial effects of genetic or pharmacological sEH inhibition as observed in various animal models of cardiovascular and inflammatory diseases (24, 25, 39, 40). Also, the enantioselectivity of mEH may provide a novel clue for understanding the role of this enzyme in human diseases (41, 42). Indeed, mEH significantly determines the endogenous levels of monoepoxides and only a sEH/mEH-double KO was sufficient for almost completely preventing their hydrolysis to the corresponding vicinal diols in mice (23). Thus, it is tempting to speculate that polymorphic variants and/or xenobiotics leading to alterations in mEH expression and activity could have an important impact on bioactive lipid mediator formation via the CYP epoxygenase pathway.

Further studies are needed to better understand the complex interactions of CYP enzymes, epoxide hydrolases, and other factors in determining the profile of regio- and stereoisomeric fatty acid epoxides under in vivo conditions. It has been suggested that mEH primarily acts as a “first-pass” hydrolase for the monoepoxides generated by the adjacent CYP enzymes in the ER, whereas the sEH comes into play after the remaining monoepoxides have been released into the cytosol (23). Accordingly, the mEH is
expected to make a significant contribution to total intracellular monoepoxide hydrolysis, in particular, under basal conditions, i.e., when monoepoxides are formed at low rates due to the limited availability of free fatty acids for CYP enzymes. The sEH may take over a predominant role after mEH capacities are overwhelmed by increased monoepoxide formation in response to physiological or pathophysiological stimuli that activate phospholipases A2 and, thereby, make free AA, EPA, and DHA accessible to the CYP enzymes. The actual impact of mEH and sEH certainly also depends on the relative expression of the two epoxide hydrolases in a given cell type (23). A general question concerns the mechanisms of how monoepoxides generated by CYP enzymes can escape, at least in part, the attack of both mEH and sEH as required to exert their biological functions and to become endogenous constituents of

Fig. 7. Role of sEH in determining the enantiomeric composition of endogenous monoepoxy metabolites in mice. Liver (A) and plasma samples (B) were prepared from WT and sEH-KO mice after the animals received a diet supplemented with EPA and DHA for 3 weeks. The enantiomeric composition of the targeted metabolites was determined using chiral-LC-ESI-MS/MS. Data are shown as mean ± SEM (n = 5–6) and represent the total levels of the metabolites (free + esterified) as accessible after alkaline hydrolysis of the samples. Statistically significant differences were observed as indicated: *P < 0.05 versus corresponding (R,S)-enantiomer and #P < 0.05 versus WT. See also supplemental Tables S5 and S6 for the enantiomeric composition of further oxylipins in liver and plasma of WT and sEH-KO mice.

Fig. 8. Profile of enantiomeric oxylipins in human plasma. Plasma samples were prepared from healthy volunteers 8 weeks after dietary EPA/DHA supplementation. The enantiomeric composition of the targeted metabolites was determined using chiral-LC-ESI-MS/MS. A: Levels of enantiomeric monohydroxy metabolites. B: Levels of enantiomeric monohydroxy metabolites. Data are shown as mean ± SEM (n = 6, three male and three female) and represent the total levels of the metabolites (free + esterified) as accessible after alkaline hydrolysis of the samples. Statistically significant excess of individual enantiomers was observed as indicated: *P < 0.05 versus corresponding (R,S)/((R)/(P1)-enantiomer.
the plasma, liver, and other tissues. Reinforcing this question, a recent study showed that tissue sEH concentrations range from 3 (lung) to 400 nM (liver), whereas the EET concentrations are generally in the low nanomolar range (43). Accordingly, it has been suggested that the intracellular hydrolysis of monoepoxides is limited by substrate accessibility rather than the amount of sEH activity (43). Providing a potential mechanism, fatty acid binding proteins (FABPs) that are highly expressed in the liver and other tissues were shown to bind EETs and to efficiently inhibit their sEH-mediated hydrolysis under in vitro conditions (44, 45). Based on the role of FABPs in intracellular trafficking and delivery of long-chain fatty acids (46, 47), it is tempting to speculate that FABPs might similarly control the compartmentalization and metabolic utilization of fatty acid monoepoxides and, thus, prevent their rapid and indiscriminate hydrolysis in the liver and other tissues. At least some of the questions discussed above can now be addressed using chiral lipidomics. In particular, it will be of interest to determine the regio- and stereoisomeric profiles of free and esterified fatty acid epoxides i) in different tissues, ii) under basal and stimulated conditions, and iii) in disease conditions associated with changes in CYP, mEH, sEH, and FABP expressions. Moreover, the efficiency and specificity of FABP-mediated protection of monoepoxides remains to be tested under in vivo conditions, e.g., by using FABP inhibitors, in order to understand the potential impact of metabolic changes that increase the levels of competing FABP ligands (45).

In line with a recent study (7), our chiral analysis showed that monohydroxy metabolites are generally present as racemates in the human circulation, whereas enantioselective responses occur after stimulating their de novo biosynthesis. Ca²⁺-ionophore stimulation resulted in specific
increases of the (S)-enantiomers of those HETEs, HPEPs, and HDHAs that were apparently produced by the stereospecific actions of 5-, 12-, and 15-LOX enzymes expressed in different blood cells (48–50). In contrast, the stimulated formation of 11(R)-HETE was most likely mediated by COX enzymes (51). Interestingly, 18-HEPE was also increasingly generated in a stereospecific manner. 18-HEPE is the precursor of resolvins, and its enzymatic versus nonenzymatic origin has not been fully clarified (8). No changes occurred with 9-HETE, a racemic monohydroxy metabolite thought to be formed by free radical oxidation reactions (52).

Taken together, we developed a reversed-phase chiral-LC-ESI-MS/MS approach that is applicable for the profiling of regio- and stereoisomeric oxylipins in biological samples. The targeted metabolites include enantiomers of monoepoxy and monohydroxy metabolites as primarily formed by CYP and LOX enzymes. Future applications of the established analytical system may help to better understand associations between changes in the endogenous formation of these lipid mediators, their stereospecific biological activities, and the development of cardiovascular and inflammatory diseases.44

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