COMPENSATORY MECHANISM FOR HOMEOSTATIC BLOOD PRESSURE REGULATION IN *EPHX2* GENE DISRUPTED MICE

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Oxylipin extraction- Oxylipins were isolated by solid phase extraction on 60 mg Waters Oasis-HLB cartridges (Milford, MA). Prior to extraction, cartridges were washed with 2 mL ethyl acetate followed by 2 mL methanol and conditioned with 2 mL of 95:5 v/v water/methanol with 0.1% acetic acid. Sample aliquots (250 µL plasma; 2 mL urine) were then introduced to the column reservoir and spiked with analytical surrogates: 10,11-dihydroxynonadecanoic acid (10,11-DHN); 10,11-epoxyheptadecanoic acid (10(11)-EpHep); tetradeuterated-6-keto-prostaglandin F1α (d4-PGF1α). Surrogates were delivered in 10 µL methanol for a final concentration of 400 nM. Overall method performance was evaluated using, phosphate buffered saline (PBS; 0.1 M phosphate buffer, 0.8% NaCl, pH 7.4) spiked with the surrogates and a subset of the target analytes covering each class of oxylipin. A PBS sample spiked only with the surrogate solution was used as an analytical reagent blank. A matrix spike and blank were included with each batch of twenty samples. Plasma samples and their associated matrix spikes and blanks were diluted 1:1 with the 95:5 v/v water/methanol with 0.1% acetic acid. Urine samples and their associated matrix spikes and blanks were extracted undiluted. Samples were then loaded onto SPE columns, and the columns were washed twice with 1.5 mL of 95:5 v/v water/methanol with 0.1% acetic acid. Excess solvents were removed by drying the SPE column under low vacuum (15 min, <20 KPa). The SPE columns were then wetted with 0.5 mL methanol and analytes eluted with 2.0 mL of ethyl acetate. Samples were collected into labeled polypropylene tubes evaporated under nitrogen and, re-suspended in 50 µl methanol containing the internal standard 1-cyclohexyl ureido,3-dodecanoic acid (CUDA) at 800 nM. Extracts were stored at -20ºC until analysis by HPLC/MS/MS. The internal standard was used to quantify the recovery of surrogate standards.

Oxylipin analysis- Analytes in a 20 µL extract aliquot were separated with the solvent gradient described in Table S1 using a Waters 2790 HPLC. Samples were held at 10ºC. Separated residues were detected by negative mode electrospray ionization and multiple reaction monitoring on a Quattro Ultima tandem

Table S1. Oxylipin HPLC parameters

Time (min)	A%	B%	Flow (mL/min)
0.0	85	15	0.30
0.5	85	15	0.35
2.0	70	30	0.35
8.0	45	55	0.35
10.5	45	55	0.35
13.0	45	55	0.35
33.0	25	75	0.35
34.0	0	100	0.35
38.0	0	100	0.35
38.1	85	15	0.35
40.0	85	15	0.35

Solvent A = 0.1% Acetic Acid; Solvent B = 88:12 Acetonitrile/MeOH w/ 0.1% Acetic Acid; column = 2.1 x 150, 5 µm Luna C18(2) (Phenomenex; Torrance, CA), column temp = 40ºC.

quadrupole mass spectrometer (Micromass, Manchester, UK) using the following operating parameters: capillary voltage = -3.2 kV; cone gas = 125 L/h; desolvation gas = 650 L/h; source temp = 100ºC; desolvation temp = 400ºC; collision gas pressure = 2.3 mTorr argon; photo

Table S2. HPLC / Electrospray ionization tandem mass spectroscopy parameters*

Analyte	tR (min)	Transition (Da)	Cone (V)	Collision (V)	Dwell (secs)	Internal Standards†
d4 6-keto-PGF1α	6.90	373.3 > 167.1	60	24	0.6	CUDA
6-keto-PGF1α	6.90	369.2 > 163.1	60	24	0.6	d4 6-keto-PGF1α
TXB2	8.07‡	369.3 > 195.2	60	22	0.4	10,11-DHN
PGF2α	8.70	353.2 > 193.1	60	23	0.4	10,11-DHN
9,12,13-TriHOME	8.76	329.2 > 211.2	60	15	0.4	10,11-DHN
9,10,13-TriHOME	8.85	329.2 > 171.1	60	15	0.4	10,11-DHN
PGE2	8.95	351.2 > 271.2	60	12	0.4	10,11-DHN
CUDA	13.35	340.3 > 214.1	60	20	0.4	---
10,11-DHHep	13.81	301.2 > 283.2	60	24	0.4	10,11-DHN
12,13-DHOME	13.81	313.2 > 183.1	60	23	0.4	10,11-DHN
9,10-DHOME	14.50	313.2 > 201.1	60	23	0.4	10,11-DHN
14,15-DHET	15.42	337.2 > 207.1	60	17	0.6	10,11-DHN
11,12-DHET	16.65	337.2 > 167.1	60	20	0.6	10,11-DHN
8,9-DHET	17.78	337.2 > 127.1	60	20	0.4	10,11-DHN
19-HETE	18.10	319.2 > 275.2	60	16	0.4	10,11-DHN
20-HETE	18.44	319.2 > 275.2	60	16	0.4	10,11-DHN
5,6-DHET	19.27	337.2 > 145.1	60	17	0.4	10,11-DHN
13-HODE	21.04	295.2 > 195.2	55	17	0.4	10,11-DHN
10,11-DHN	21.10	329.2 > 311.2	55	26	0.4	CUDA
9-HODE	21.27	295.2 > 171.1	55	17	0.4	10,11-DHN
15-HETE	22.23	319.2 > 219.1	55	13	0.4	10,11-DHN
13-oxo-ODE	22.40	293.2 > 195.2	60	23	0.4	10,11-DHN
11-HETE	23.23	319.2 > 167.1	55	14	0.35	10,11-DHN
9-oxo-ODE	23.40	293.2 > 185.1	55	22	0.35	10,11-DHN
12-HETE	23.91	319.2 > 179.1	55	14	0.35	10,11-DHN
8-HETE	23.97	319.2 > 155.1	55	14	0.35	10,11-DHN
9-HETE	24.59	319.2 > 123.1	55	14	0.35	10,11-DHN
5-HETE	25.27	319.2 > 115.1	55	14	0.35	10,11-DHN
10(11)-EpHep	26.18	283.2 > 185.1	55	20	0.5	CUDA
12(13)-EpOME	26.25	295.2 > 195.1	55	17	0.5	10(11)-EpHep
14(15)-EET	26.75	319.2 > 219.1	55	13	0.5	10(11)-EpHep
9(10)-EpOME	26.79	295.2 > 171.1	55	17	0.5	10(11)-EpHep
11(12)-EET	28.25	319.2 > 208.1	55	13	0.5	10(11)-EpHep
8(9)-EET	28.87	319.2 > 155.1	55	13	0.5	10(11)-EpHep
5(6)-EET	29.33	319.2 > 191.1	55	10	0.5	10(11)-EpHep

* - Analytes were separated under conditions described in Table I. Collision-induced dissociation was performed with argon at a pressure of 2.3 mTorr. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

† - Analytes were corrected for recoveries of listed surrogates. 1-Cyclohexylureido,3-dodecanoic acid (CUDA; CAS# 479413-68-8) was introduced immediately prior to analysis and used to quantify surrogate recoveries.

‡ - The chromatography of TXB2 under the described conditions produces two peaks (initial shown) separated by a saddle. The secondary apex was at 9.01 min.

multiplier voltage = 650. Analyte retention times, mass transitions, optimized cone and collision voltages, dwell times, and analytical surrogate associations for each analyte are shown in Table S2. Ion dwell times yielded a minimum of eight scans across the chromatographic peaks.

Analytes were quantified using internal standard methods and five point calibration curves fit with 1/x weighted quadratic curves ($r^2 \geq 0.997$). Calibrants and internal standards were either synthesized [10,11-DHN, 10,11-DHHep, 10(11)-EpHep and CUDA] or purchased from Cayman Chemical (Ann Arbor, MI) unless otherwise indicated. Lordan Fine Lipids (Malmo, Sweeden) provided the linoleate derived triols 9,12,13-TriHOME and 9,10,13-TriHOME. With the exception of d4-PGF1α in urine, surrogate recoveries were acceptable during this study (Table S3).

Table S3. Oxylipin surrogate recoveries.

	10,11-DHN	**10(11)-EpHep**	**d4-6-keto-PGF1α**
Plasma	64 ± 8%	71 ± 4%	76 ± 5%
Urine	88 ± 5%	99 ± 7%	25 ± 2%

SUPPLEMENTAL RESULTS

Principal components analysis- To highlight the changes observed in the described oxylipin profiles, the data were analyzed by principal components analysis (PCA). This analysis provides a complimentary view of the tabular data reported in the primary text. Briefly, PCA seeks to reduce the total number of variables to the fewest number in order to describe the maximum amount of variance in the data set while providing a weight for each variables impact on the over all variance. As shown in Fig. S1, a clear separation between the genotypes was found by the PCA analysis of plasma, tissue homogenate, and urine results. In *Ephx2*-null mouse plasma the P450 epoxygenase pathway was elevated while the ω-hydroxylase metabolite 20-HETE was not detected. This separation was primarily driven by the epoxides of linoleate. In kidney and liver homogenates, the arachidonic acid metabolite production also segregated the wild type and *Ephx2*-null phenotypes. While diol production was reduced and epoxide accumulation was increased in both tissue homogenates, other enzyme systems were differentially modified. Moreover, this analysis clearly shows the additional metabolite shifts occurring in the kidney, with a significant increase in 20-HETE production. Urinary oxylipin analyses also discriminated the *Ephx2*-null and wild type genotypes suggested general trends toward increased epoxides and 20-HETE, and decreased diols and 12-HETE in the urine of *Epxh2*-null mice (Fig. S1).

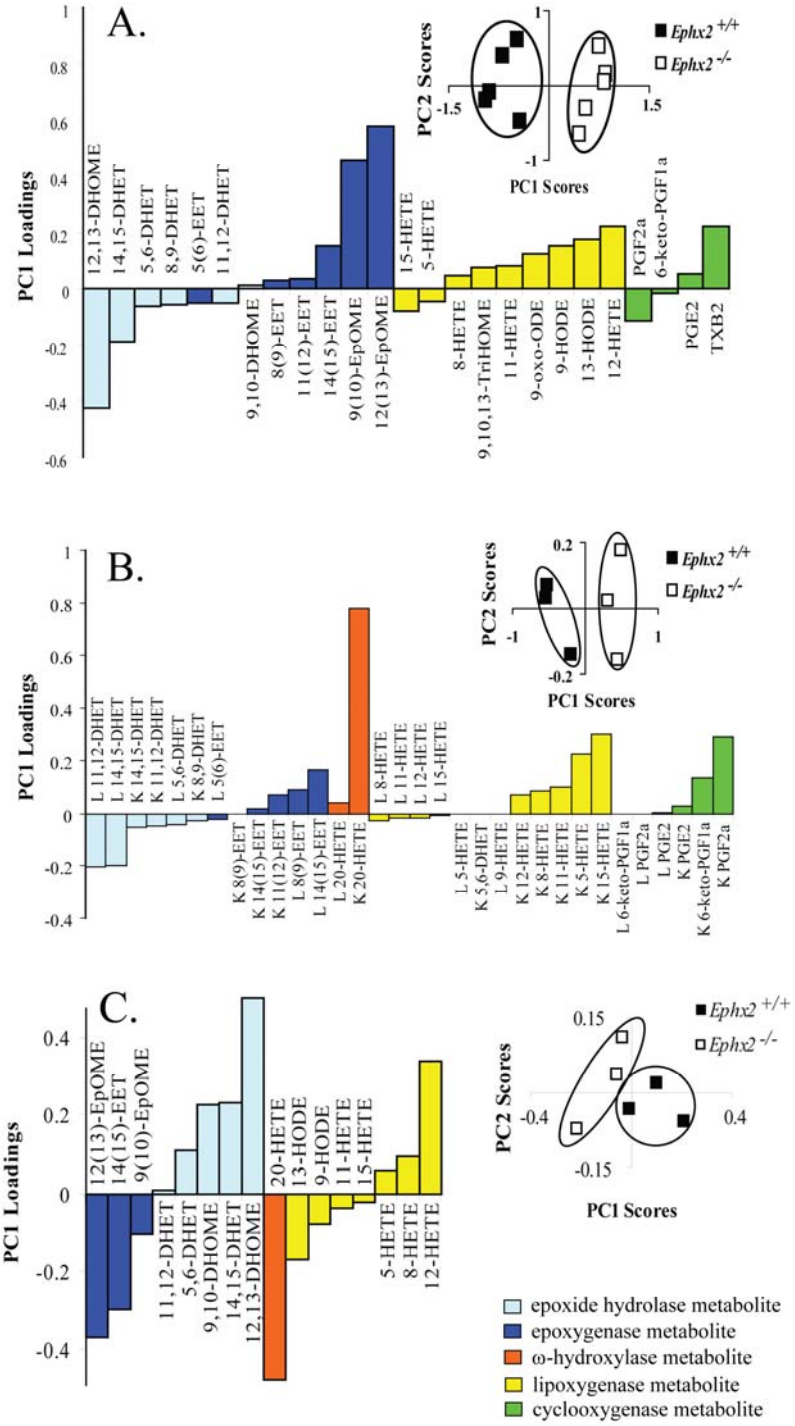

Fig. S1. Principal components (PC) analyses of (**A**) plasma oxylipins (nM), (**B**) tissue oxylipin production (fmol/min/mg protein) and (**C**) urine oxylipins (nM). Results summarized from BI colony were mean centered and standardized to unit variance before analysis. The loading plots describe the weight that each variable has in separating samples in the scores plots (insets).