Supporting Information

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SI Materials and Methods

Animals and Vessels. Twelve- to 15-wk-old male and female C57BL/6J mice were received from the Jackson Laboratories. Some female mice were ovariectomized at 9 wk of age. One week after ovariectomy, the mice were randomly divided into two groups. One group received a subcutaneous injection of vehicle (OV) and the other received 17β-estradiol benzoate injections (OVE, 50 μg/kg, every 48 h; Sigma) for 3 wk (1, 2). Mice were killed by inhalation of carbon dioxide (CO_2) and the mesenteric arteries were isolated, with each sample consisting of ~50% of the total mesenteric arteries isolated from one mouse. In separate experiments, isolated aortic and carotid arteries combined with mesenteric arteries were also used. Mesenteric arteries were selected because of their abundance and sufficient length. Moreover, the sex difference in the vascular sEH protein expression, followed by corresponding changes in vascular function and mediators, have been extensively reported in our previous publications (3–8).

Vessel Cultures. Isolated vessels from male mice were placed in a 12-well dish filled with DMEM (Cellgro), supplemented with 10% FBS (vol/vol) and 1% antibiotics (Antimycin solution 100×), and incubated in the absence (as controls) or presence of 17β-estradiol (17β-E_2; 10 nM; Sigma) under a 5% CO_2 atmosphere at 37 °C for 24, 48, and 72 h, respectively. DMEM containing 17β-E_2 was prepared fresh and replaced on a daily basis.

All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals.

Cell Cultures. Partial studies were performed on the human cell line HEK293T, purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics under a 5% CO_2 atmosphere at 37 °C, and passaged at 90–100% confluency.

qRT-PCR. Total RNA was extracted from isolated mesenteric arteries using TRIzol solution (Sigma-Aldrich) and treated with RNase-free DNase. Total RNA (2 μg) from each sample was reverse-transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase (Promega), with a reverse-transcriptase negative control reaction. qPCR analysis was performed to compare relative quantities of gene expression by employing a LightCycler system (Roche Diagnostics), according to the manufacturer's instructions and a QuantiTect SYBR Green PCR kit (Qiagen). The primers were designed in-house (Table S1) and synthesized by Fisher Scientific customer services. Quantification was reported, using proprietary software, as cycle threshold (Ct). The sEH expression was normalized to GAPDH or actin. A relative quantitation method (ΔΔCt) was used to evaluate the expression of the gene in different groups of samples. All amplified products were verified on a 1.5% agarose gel.

BGS and MSP. Bisulfite modification was performed as described previously (9). Specifically, genome DNA of vessel samples was modified via treatment with sodium bisulfite to convert unmethylated, but not methylated, cytosines to uracils (EpiTectPlus DNA Bisulfate Kit; Qiagen), and the modified DNA was used as a template for PCR. The primers for MSP and BGS amplification were designed using the bioinformatics program (www.urogene.org/methprimer/) and according to the CpG islands in the *Ephx2* gene promoter region (Table S1). The MSP was performed using the primers that are specific for the methylated or unmethylated DNA sequence. PCR products were visualized by separation on agarose gels and stained with ethidium bromide. The density of the specific band with appropriate length indicates the quantity of unmethylated or methylated alleles in the original sample. The methylation expression was calculated by the ratio of methylated/unmethylated CpG sites (CpG/TpG × 100) from each mouse arterial DNA, thus providing a percentage of methylation from the total CpG sites. The BGS assay involved two PCR amplificons in *Ephx2* promoter region: the proximate one from −217 to +57 bp with 21 CpG sites and the distal one from −970 to −427 bp with four CpG sites. The sequencing was performed by W. M. Keck Biotechnology Resource Laboratory, Yale School of Medicine.

Luciferase Activity Assay. Two reporter plasmids were constructed with two human *Ephx2* promoter regions, the −374 to +28 region (402 bp) and −123 to +28 region (151 bp), cloned immediately upstream of the firefly luciferase gene in the pGL3-Basic reporter plasmid (Promega) (10), respectively. Each construct was confirmed by sequencing.

Luciferase activity was assessed based on a previous published method (10). Briefly, HEK293T cells were seeded in 24-well culture plates at a density of 1×10^5 cells per well and left to recover for 24 h, and then, transfected with 0.5 μg of each single plasmid using the Lipofectamine 3000 protocol provided by the manufacturer. For each transfection, 10 ng of pRL-CMV (Promega), a plasmid that expresses *Renilla* luciferase under the control of an acytomegalovirus (CMV) promoter, was given as a cotransfected internal control reporter for transfectional efficiency. The cells were transfected with either a pGL3-Basic reporter plasmid as a negative control or pGL3 with CMV promoter as positive control, or pGL3 with sEH promoters (0.4 and 0.12 kb, respectively). After transfection for 24 h, cells were treated with 17β-E_2 (10 nM) or 17β-E_2 plus ICI 182,780 (ER antagonist; 100 nM; Tocris Bioscience), respectively, for 48 h. Following incubation, cells were collected and lysed with 100 μL per well of 1× Passive Lysis Buffer (Luciferase Reporter Assay Kit). Five microliters of cell lysate was used for each reaction of the luciferase assay. The luciferase assay was performed by using the Dual-Glo Luciferase Reporter Assay System (Promega). Luciferase activities were determined using a 20/20 Luminometer (Turner BioSystems, Fisher Scientific). Firefly luciferase activity was normalized to the internal control (pRL-CMV) and expressed as the ratio of luciferase activity/*Renilla* luciferase activity.

ChIP assay. ChIP assay was performed based on the protocol of cross-linking ChIP (Abcam). Due to concerns of tissue abundance necessary for performing the assay, mouse vessel samples were combined with aorta, carotid, and mesenteric arteries together and homogenized in DMEM. Homogenized tissue/cells were cross-linked with 0.75% formaldehyde at 37 °C for 10 min. Following fixation, the tissue/cells were rinsed with PBS, and centrifuged to obtain the pellets. The pellets were lysed in SDS lysis buffer with 10-min incubation on ice, and then sonicated 20 s three times, aimed to break the chromosomal DNA into 1-kb fragments or smaller. The lysate was then centrifuged (10,000 × *g* for 10 min at 4 °C) and the supernatant fraction was collected and diluted in 1 mL ChIP dilution buffer. Twenty-five microliters of diluted supernatant was reverse–cross-linked, followed by DNA purification and determination of DNA amount. Thirty micrograms of DNA from each sample was used for immunoprecipitation probed with

2 μg of antibodies (anti-SP1, anti–AP-1, anti–NF-κB, or control rabbit IgG; Santa Cruz Biotech). The reaction was processed overnight at 4 °C. Immunocomplexes were extracted from the immunoprecipitation reaction samples by the addition of 20 μL protein A/G agarose and incubated for an additional 1 h at 4 °C. The precipitates were sequentially washed with low-salt wash buffer, high-salt wash buffer, and LiCl wash buffer (1 mL for each), respectively. The immunocomplexes were eluted with 120 μL elution buffer (1% SDS, 0.1 M $NaHCO_3$) and shaken for 15 min at 30 °C. To remove the DNA cross-links, 4.8 μL of 5 M NaCl was added to elutes, which was then heated overnight at 65 °C. After that, 2 μL of 20 mg/mL proteinase K was added, followed by incubation at 45 °C for an additional 1 h. The released DNA fragments were then phenol extracted and alcohol-precipitated. Purified DNA fragments were used as a template for qPCR. The primers (Table S1) were used to amplify the −212- to −73-nt region on the mouse *Ephx2* promoter, which is in the vicinity of all of the transcriptional factor binding elements to be investigated: one SP1 binding element (located inside of the PCR product); two AP-1 binding elements (one 680-bp upstream and the other 38-bp downstream), and two NF-κB binding elements (one within the amplicon and the other in 55-bp upstream). After immunoprecipitation and DNA purification, DNA quantitation was determined by qPCR.

For the human *Ephx2* promoter, 293T cells (1×10^6 cells) were seeded into 10-cm-diameter tissue-culture plates overnight, and then incubated with or without 17β-E_2 (10 nM), 17β-E_2 plus ICI 182,780 (100 nM), or 17β-E_2 plus Decitabine (4 μM; DNA methyltransferase/DNMT inhibitor; Fisher Scientific) for 48–72 h, and then cells/samples were collected. After immunoprecipitation, purified DNA fragments were used as a template for qPCR that amplify the −214- to +28-nt (from start codon) fragment of the 5′-flanking region of the human *Ephx2* gene (10).

Statistical analysis. Data are expressed as means ± SEM. n refers to numbers of mice or PCR reactions. Statistical analysis was performed using repeated-measures ANOVA followed by the Tukey–Kramer post hoc test and Student's t test. Statistical significance was accepted at a level of $P < 0.05$.

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Table S1. Primers for mouse *Ephx2* gene

Primer type	Primer	Primer sequence 5′ 3′	Product, bp	Annealing, °C
RT-PCR primers (mouse Ephx2)	mEphx2 F	CTGTGGCCAGTTTGAACACG	179	55
	mEphx2 R	ATCACTGGCTCGGAAGAAGC		
RT-PCR primers (mouse GAPDH)	mGAPDH F	CACTCTTCCACCTTCGATGCC	240	55
	mGAPDH R	CTGGGATGGAAATTGTGAGGG		
MSP primers (CpG −13/−15)	meF-13	TTTATTGATTAGTTG TTTAGTTCGG	221	53
	meR-13	AAAAAAAATATTTAAAATATTCCGTC		
	umF-13	TTATTGATTAGTTGTTTAGTTTGG		
	umR-13	AAAAAAATATTTAAAATATTCCATC		
MSP primers(CpG −17/−18)	meF-17	TCTCTACTAATGAGTTAATTAACAAATCTATC	102	50.1
	meR-17	AAACAAAAAAAAAAGCCAATTCCTG		
	umF-17	TCTCTACTAATGAGTTAATTAACAAATCTATT		
	umR-17	AAACAAAAAAAAAAGCCAATTCTCA		
BGS primers (proximate end sequencing)	6F	GATAGAGGGGAGGGTTAGTATTT	273	55
	6R	AATAAAACATCCAACAAAAAACTCC		
BGS primers (distal end sequencing)	549F	GTTGGGATTTGTGGATTTTTTTCTT	546	55
	549R	CCCACATTTTTAACCACTTTCCA		
Mouse ChIP primers	mChIP F	GGCTGTGGGAACCTAAGA	140	51
	mChIP R	GACATCCAGCAAGAAGCTC		