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Soluble epoxide hydrolase null mice exhibit female and male differences in regulation of vascular homeostasis

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ABSTRACT

Increased CYP epoxygenase activity and consequently up regulation of epoxyeicosatrienoic acids (EETs) levels provides protection against metabolic syndrome and cardiovascular diseases. Conversion of arachidonic acid epoxides to diols by soluble epoxide hydrolase (sEH) diminishes the beneficial cardiovascular properties of these epoxyeicosanoids. We therefore examined the possible biochemical consequences of sEH deletion on vascular responses in male and female mice. Through the use of the sEH KO mouse, we provide evidence of differences in the compensatory response in the balance between nitric oxide (NO), carbon monoxide (CO), EETs and the vasoconstrictor 20-HETE in male and female KO mice. Serum levels of adiponectin, TNFα, IL-1b and MCP1 and protein expression in vascular tissue of p-AMPK, p-AKT and p-eNOS were measured. Deletion of sEH caused a significant ($p < 0.05$) decrease in body weight, and an increase in adiponectin, pAMPK and pAKT levels in female KO mice compared to male KO mice. Gene deletion resulted in a higher production of renal EETs in female KO compared to male KO mice and, concomitantly, we observed an increase in renal 20-HETEs levels and superoxide anion production only in male KO mice. sEH deletion increased p-AKT and p-eNOS protein expression but decreased p-AMPK levels in female KO mice. Increased levels of p-eNOS at Thr-495 were observed only in KO male mice. While p-eNOS at 1177 were not significantly different between male and female. Nitric oxide production was unaltered in male KO mice. These results provide evidence of gender differences in the preservation of vascular homeostasis in response to sEH deletion which involves regulation of phosphorylation of eNOS at the 495 site.

1. Introduction

Vascular endothelial cells play a crucial role in vascular physiology and pathophysiology through the release of various vasoactive autacoids. Nitric oxide (NO), the best known of these autacoids, maintains vascular homeostasis and also protects vessels from injuries [1–4]. Cardiovascular and related disorders are frequently associated with endothelial dysfunction exemplified by the impairment of NO formation [5–7]. In vascular endothelium, NO is produced by the constitutively expressed enzyme endothelial nitric oxide synthase (eNOS) which catalyzes the conversion of L-arginine to L-citrulline and NO, the latter mediates a variety of actions such as vasodilatation, neurotransmission and host defense against bacteria and tumor cells [8–10]. NO is a regulator of angiogenesis which enhances vascular permeability, induces extracellular matrix degradation, increases endothelial cell proliferation and migration and stimulates the expression of vascular growth factor (VEGF) [11]. The endothelium is also a major source of critical vasodilators such as epoxyeicosatrienoic acids (EETs) which benefit the endothelium by regulating vascular tone, coagulation, smooth muscle cell proliferation and apoptosis [12]. Epoxidation of arachidonic acid to EETs is catalyzed by a number of cytochrome P450 (CYP) isoforms that demonstrate tissue-specific expression. Members of the CYP2C and CYP2J families are the predominant epoxygenases in liver, kidney, brain and blood vessels with important biological and vascular functions in both rodents and humans

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[13–15]. Mice with elevated levels of EETs exhibit lower blood pressure than age matched WT control mice and upregulation of EETs levels provides protection against Ang II-induced vascular injury [16].

The arachidonic acid metabolites of CYP-epoxygenases, that include 5,6- 8,9- 11,12- and 14,15-EETs, are rapidly degraded and inactivated by soluble epoxide hydrolase (sEH) which is present in a number of mammalian tissues, including the liver, kidney, intestine and blood vessels. Within these tissues, sEH metabolizes epoxide-containing compounds to their corresponding diols. Conversion of arachidonic acid epoxides to diols diminishes the beneficial cardiovascular properties of EETs. Inhibition of sEH causes EETs to accumulate and be retained in tissues for extended periods after they are formed [17,18]. In addition to their vasodilatory activity and anti-hypertensive actions, EETs inhibit platelet aggregation, promote fibrinolysis and have anti-inflammatory properties [19–21]. Numerous studies have demonstrated that increased EETs production and levels by induction of CYP-epoxygenases, administration of EET analogs and inhibition of sEH are beneficial in the treatment of cardiovascular diseases [22].

A role for CYP-derived eicosanoids in the regulation of blood pressure is well established. In particular, kidney and vascular endothelium produce significant levels of CYP-derived eicosanoids which affect both renal tubular transport function and vascular reactivity [23,24]. 20-Hydroxyeicosatetraenoic acid (20-HETE) is the ω-hydroxylation metabolite of arachidonic acid formed in vascular tissues by members of the CYP4A and CYP4F families. 20-HETE is primarily found in the microcirculation of the kidney, liver and brain. 20-HETE is a pro-hypertensive lipid autacoid and a potential mediator of androgen-induced hypertension [25,26]. The interplay between epoxygenases and ω-hydroxylases in the kidney and vasculature is an important determinant of blood pressure control. Altered levels of renal EETs and 20-HETE are associated with perturbations in blood pressure and are gender-specific [27–32].

The current study examines the impact of sEH deletion on biochemical and molecular indices of vascular function and assesses whether these changes are gender specific. Silencing sEH upregulates renal EETs levels and increases the phosphorylation of vascular AKT (Ser473) and eNOS at both Ser-1177 and Thr-495. We provide evidence of the existence of a different compensatory response in the balance between NO, EETs and 20-HETEs in male and female KO mice, through studies in the sEH KO mouse,

2. Material and methods

2.1. Animal protocols

Male and female sEH null mice, 12 weeks old, were provided by Dr. Darryl C. Zeldin, Division of Intramural Research, National Institute of Environmental Health. Aged and sex-matched B6129SF2/J mice served as controls. Mice were fed a normal laboratory chow diet and had free access to water and food. There was no difference in food intake in any of the treatment groups. Cobalt protoporphyrin IX (CoPP) (3 mg/kg once a week) was administered i.p. for 8 weeks. The Marshall University Institutional Animal Care and Use Committee approved all the experiments in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals.

2.2. Tissue preparation and Western blot analysis

At sacrifice, aorta and kidney tissues were immediately collected, weighed and frozen at −80 °C until use. Tissues were homogenized (4 ml/g wet weight) in a homogenization buffer (pH 7.4) consisting of 0.25 M sucrose, 0.5% Nonidet P-40 and 10 mM EDTA in TBS (20 mM Tris and 150 mM NaCl, pH 7.4) containing a cocktail of protease inhibitors (Sigma–Aldrich, St. Louis, MO) and Halt™, a phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL). The homogenates were centrifuged at 10,000 × *g* for 10 min at 4 °C.

The cell-free homogenate (10,000 × *g* supernatant) was used for Western blot analyses and activity assays. Protein concentration was determined using the Bradford method (BioRad, Hercules, CA). Western blot analysis of protein expression was performed as previously described [33]. Briefly, cell-free homogenates (10,000 × *g* supernatant) of aorta preparations (20 μg protein) were separated by SDS/polyacrylamide gel electrophoresis and transferred to a PVDF Immobilon-P membrane (Amersham Pharmacia, Piscataway, NJ) using a semidry transfer apparatus (Bio-Rad, Hercules, CA). The membranes were incubated with 5% milk in 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20 (TBST) buffer at 4 °C overnight. After washing with TBST, the membranes were incubated with primary antibodies. Antibodies against eNOS and p-eNOS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The membranes were then washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG. Chemiluminescence detection was performed using the Amersham ECL detection kit according to the manufacturer's instructions (Amersham Pharmacia, Piscataway, NJ).

2.3. Determination of EETs and DHETs levels

EETs and DHETs levels were measured in kidney tissues using liquid chromatography–tandem mass spectrometry. Briefly, renal cortical tissues were homogenized in 66% methanol containing a 500-pg mixture of internal standards. EETs were extracted using solid phase C18-ODS AccuBond II 500-mg cartridges. The collected methanol fraction was dried under nitrogen and then resuspended in 200 μl of methanol and stored at −80 °C until analysis by liquid chromatography–tandem mass spectrometry. Synthetic standards were used to obtain standard curves (5–500 pg) for each compound. The standard curves were used to calculate the final EET and DHET concentrations, expressed as nanograms per milligram of protein.

2.4. Measurement of O_2^- levels in aorta and kidney tissues

Samples were placed in plastic scintillation minivials containing 5 mm lucigenin for the detection of O_2^- in a final volume of 1 ml of air-equilibrated Krebs solution buffered with 10 mM HEPES-NaOH (pH 7.4). Lucigenin chemiluminescence was measured in a liquid scintillation counter (LS6000IC; Beckman Instruments, San Diego, CA) at 37 °C; data are reported as counts/min/mg protein after background subtraction.

2.5. Cytokine measurements

Tumor necrosis factor alpha (TNFα), monocyte chemotactic protein-1 (MCP-1), interleukin-1 beta (IL-1β) and adiponectin (high molecular weight, HMW), were measured as previously described [34,35] by Cytokine SearchLight Infrared arrays (Pierce Biotechnology, Inc., Woburn, MA, USA).

2.6. NO_2^-/NO_3^- measurement

Nitrite, the stable metabolite of NO, was measured colorimetrically by the Griess reaction. Aliquots of homogenates were pre-incubated for 30 min at room temperature with 50 μmol/L NADPH (Sigma–Aldrich, St. Louis, Mo, USA) and 24 mU of nitrate