Kidney

Orally Active Epoxyeicosatrienoic Acid Analog Attenuates Kidney Injury in Hypertensive Dahl Salt–Sensitive Rat

Md. Abdul Hye Khan, Jan Neckář, Vijay Manthani, Ramu Errabelli, Tengis S. Pavlov, Alexander Staruschenko, John R. Falck, John D. Imig

Abstract—Salt-sensitive hypertension leads to kidney injury. The Dahl salt–sensitive hypertensive rat (Dahl SS) is a model of salt-sensitive hypertension and progressive kidney injury. The current set of experimental studies evaluated the kidney protective potential of a novel epoxyeicosatrienoic acid analog (EET-B) in Dahl SS hypertension. Dahl SS rats receiving high-salt diet were treated with EET-B (10 mg/kg per day) or vehicle in drinking water for 14 days. Urine, plasma, and tissue samples were collected at the end of the treatment protocol to assess kidney injury, oxidative stress, inflammation, and endoplasmic reticulum stress. EET-B treatment in Dahl SS rats markedly reduced urinary albumin and nephrin excretion by 60% to 75% along with 30% to 60% reductions in glomerular injury, intratubular cast formation, and kidney fibrosis without affecting blood pressure. In Dahl SS rats, EET-B treatment further caused marked reduction in oxidative stress with 25% to 30% decrease in kidney malondialdehyde content along with 42% increase of nitrate/nitrite and a 40% reduction of 8-isoprostane. EET-B treatment reduced urinary monocyte chemoattractant protein-1 by 50% along with a 40% reduction in macrophage infiltration in the kidney. Treatment with EET-B markedly reduced renal endoplasmic reticulum stress in Dahl SS rats with reduction in the kidney mRNA expressions and immunoreactivity of glucose regulatory protein 78 and C/EBP homologous protein. In summary, these experimental findings reveal that EET-B provides kidney protection in Dahl SS rats by reducing oxidative stress, inflammation, and endoplasmic reticulum stress, and this protection was independent of reducing blood pressure. (Hypertension. 2013;62:905-913.) • Online Data Supplement

Key Words: arachidonate epoxygenase ■ glomerular necrosis ■ hypertension ■ inflammation ■ oxidative stress

A substantial proportion of individuals with hypertension in industrialized countries consume high amounts of salt, and ≈50% of individuals with essential hypertension are salt sensitive, which is accompanied by progressive renal damage.1–4 The underlying mechanism of salt-sensitive hypertension and the associated renal injury is not yet clearly known; however, it is believed to be strongly associated with elevated oxidative stress and inflammation.5–7

A number of studies demonstrated that arachidonic acid metabolites of cytochrome P450 (CYP) epoxygenase, epoxyeicosatrienoic acids (EETs), possess anti-inflammatory and antioxidative activities providing strong organ protection in a number of pathologies including salt-sensitive hypertension.8–10 Inhibition of soluble epoxide hydrolase (sEH, Ephx2), which hydrolyzes EETs to their less biologically active dihydroyeicosatrienoic acid metabolite, increases EETs bioavailability and provides kidney protection in a number of preclinical rodent models of human disease.11–13 These studies have clearly demonstrated that the kidney protection achieved by pharmacological inhibition of sEH or genetic disruption of Ephx2 is strongly associated with the antioxidative and anti-inflammatory effects of EETs.13,14

Because of promising biological actions of EETs in a number of preclinical disease models, interest in developing EET-based therapeutic strategies has grown enormously, and that inspired the development of sEH inhibitors. Because sEH metabolizes EETs to their less active diols, sEH inhibition, in principle, indirectly increases endogenous EET levels. But the major limitations of sEH inhibitors are that they result in a generalized increase in EETs and that their therapeutic effectiveness depends on CYP epoxygenase–mediated EET generation.9,10 This is an important limitation because renal and cardiovascular diseases are associated with impaired epoxygenase generation of EETs.5–10 Thus, if CYP epoxygenase–mediated EET generation is impaired in a pathological condition, then sEH inhibition will have a negligible effect to increase EET levels. It is further known that endogenously produced EETs are chemically and metabolically labile that...
could limit their therapeutic potential.\textsuperscript{15,16} As such, attempts have been made to develop EET analogs that possess EET-mimetic activity along with several key features important for stability and bioavailability.\textsuperscript{17,18} Several recently developed EET analogs have demonstrated a number of biological actions including organ protection.\textsuperscript{3,18} Although demonstrating great potential for organ protection, these previously developed EET analogs could not be administered orally.

In the present study, we have investigated the kidney-protective effect of a novel orally active EET analog in a widely used model of salt-sensitive hypertension, the Dahl salt-sensitive (Dahl SS) rat. The Dahl SS rat has been widely used to delineate the molecular mechanisms underlying the development of salt-sensitive hypertension and to evaluate the efficacy of pharmacological interventions to lower blood pressure and prevent progressive kidney injury.\textsuperscript{19,20} In the current study, we determined that a novel orally active EET analog provides marked kidney protection in Dahl SS rats without affecting blood pressure. Experimental studies provide additional evidence that the kidney protective effects of the EET analog were associated with its antioxidative, anti-inflammatory, and anti-endoplasmic reticulum (anti-ER) stress properties.

**Materials and Methods**

**Materials**

All chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO) unless otherwise mentioned. EET analog, EET-B [\(\text{N-(5-((2-acetamidobenz[d]thiazol-4-yl)oxy) penty}-\text{N-isopropylheptanamide})\)], was synthesized in the laboratory of J.R.F. The structure of EET-B provided in Figure 1A and the detailed description of the chemical synthesis process of EET-B have been described elsewhere.\textsuperscript{11} The EET antagonist, 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE), was also synthesized in the laboratory of J.R.F.\textsuperscript{21,22}

**Animals**

The Medical College of Wisconsin Institutional Animal Care and Use Committee according to the National Institutes of Health Guidelines for care and use of laboratory animals approved all animal studies. Male C57BL/6 mice (20–35 g) purchased from Charles River Laboratories (Wilmington, MA) were used for vascular reactivity studies. Mice were housed in the Biomedical Resource Center at the Medical College of Wisconsin with a 12-hour light-dark cycle and free access to water and food. To determine the kidney protective and antihypertensive effect of EET analog in salt-sensitive hypertension, sets of 6- to 8-week-old male Dahl salt-sensitive or Dahl/SS (SS/ JHsdMcwiCrl) and SSBN13 rats (SS-Chr13\textsuperscript{BN/McwiCrl}) were purchased from Charles River Laboratories. Rats were housed in the Biomedical Resource Center at the Medical College of Wisconsin with a 12-hour light-dark cycle and free access to water, and all animals were given a high-salt diet (HS) containing 8% NaCl starting on day 0 of the experimental protocol (Harlan Teklad, Madison, WI). Experimental studies were divided into the following groups: Group 1, SS13BN rats; Group 2, Dahl SS rats administered vehicle (0.05% ethanol and 0.1% PEG–400) in drinking water ad libitum for 2 weeks; Group 3, Dahl SS rats administered EET analog EET-B at a dosage of 10 mg/kg per day in drinking water ad libitum for 2 weeks. The dose of EET-B is selected from earlier studies with EET analogs performed in our laboratory.\textsuperscript{24,25}

Rats were trained for tail-cuff plethysmographic measurement of blood pressure for 5 days before the actual measurement of systolic blood pressure (SBP) on days 0, 7, and 14 of the treatment protocol. On days 0, 7, and 14, SBP was measured exactly at the same time of the day (9–10 am) in every occasion.

**Vascular Reactivity**

The vasodilator property of EET-B was determined in isolated mesenteric resistance arteries in the absence and presence of EET antagonist 14,15-EEZE. Please see the online-only Data Supplement for details of the vascular reactivity study.

**Biochemical Analysis**

At the end of the 14-day experimental period, urine, plasma, and kidney tissue samples were collected for biochemical analysis. A number of renal injury markers along with markers of oxidative stress, inflammation, and ER stress were studied. Please see the online-only Data Supplement for details of the biochemical analysis.

**Kidney Histology and Immunohistochemical Analysis**

Histology and immunohistochemical analysis were performed using protocols provided by the manufacturer and our earlier published studies.\textsuperscript{19,20,25,26} Please see the online-only Data Supplement for details of the histological and immunohistochemical analysis.

**Real-Time Polymerase Chain Reaction Analyses**

Real-Time polymerase chain reaction analysis was performed to assess the mRNA expression of inflammatory (tumor growth factor-$\beta$1) and ER stress (glucose regulatory protein 78 [GRP 78] and CCAAT/enhancer binding protein [C/EBP] homologous protein [CHOP]) markers. Please see the online-only Data Supplement for details of the real-time polymerase chain reaction analysis.
Endothelial Cell Culture Studies
Human umbilical vein endothelial cells were used to determine the anti-inflammatory activity of EET-B in the absence and presence of EET antagonist 14,15-EEZE. Please see the online-only Data Supplement for detailed methods.

Na⁺ Transport Studies
The effect of EET-B on sodium (Na⁺) transport was determined by studying its action on transepithelial Na⁺ current in immortalized mouse cortical collecting duct (mpkCCD₉₋₁) principal cells. Please see the online-only Data Supplement for detailed methods.

Statistical Analysis
Data are expressed as mean±SE and were analyzed using 1-way ANOVA followed by Tukey post hoc test for multiple group comparisons using Prism version 4.0 software by GraphPad Software Inc. (La Jolla, CA). Statistical significance was assumed at \(P<0.05\).

Results

EET-B Vasodilatory and Anti-Inflammatory Actions Are Blocked by EET Antagonism
Mesenteric resistance artery dilation to EET-B was assessed in the presence and absence of EET antagonist, 14,15-EEZE. Mesenteric resistance artery diameter was not different between experimental groups averaging 161±25 and 142±33 µm. EET-B resulted in a concentration-dependent mesenteric resistance artery dilation, reaching a maximum dilation of 42±2%. The EET antagonist, 14,15-EEZE, abolished the EET-B–mediated dilation of the mesenteric resistance (Figure 1B). Vasodilation to the NO donor, sodium nitroprusside (100 µmol/L), was similar in the absence (54±10%) or in the presence of 14,15-EEZE (59±11%).

To provide further evidence that EET-B is acting as an EET analog, we determined the anti-inflammatory activity of EET-B in cultured endothelial cells. EET-B significantly reduced the increase in endothelial cell monocyte chemotactic protein-1 (MCP-1) levels evoked by tumor necrosis factor-α. The ability of EET-B to reduce endothelial cell MCP-1 levels was abolished by the presence of 14,15-EEZE (Figure S1 in the online-only Data Supplement).

EET-B Treatment Did Not Lower Blood Pressure in Dahl SS Rats
Baseline SBP of Dahl SS (119±2 mm Hg) and SSBN13 (112±2 mm Hg) rats were similar at day 0. Dahl SS rats receiving high-salt diet developed hypertension by day 7 with significantly higher SBP compared with SSBN13 rats (172±14 versus 128±9 mm Hg) and remained elevated on day 14 (184±9 versus 131±8 mm Hg). EET-B treatment did not lower SBP in Dahl SS rats on day 7 (163±14 mm Hg) or on day 14 (183±4 mm Hg). Urine volume averaged 70±10 in SSBN13, 61±8 in Dahl SS, and 60±5 mL/d in Dahl SS + EET-B on day 14. EET-B treatment did not alter sodium excretion in Dahl SS rats, with urinary sodium excretion averaging 28±7 in SSBN13, 15±3 in Dahl SS, and 16±1 mmol/d in Dahl SS + EET-B groups. In relation to these findings, experiments were performed to determine the effect of EET-B on sodium transport using isolated cortical collecting duct cells. EET-B did not significantly reduce the short-circuit current (\(I_{sc}\)) in mpkCCD₉₋₁ principal cells, hence indicating that EET-B did not alter transepithelial sodium transport (Figure S2).

EET-B Treatment Reduced Kidney Injury in Dahl SS Rats
Dahl SS rats had marked kidney injury with 5-fold increase in urinary albumin to creatinine ratio along with tubular injury manifested by vacuolation and desquamation of the renal epithelial cells along with intratubular proteinaceous cast formation involving 5% and 10% of the kidney cortical and medullary regions, respectively. EET-B treatment resulted in marked reductions of albumin to creatinine ratio and tubular cast formation in Dahl SS rats (Figure 2A–2C). In Dahl SS rats, the kidney injury is also manifested by marked nephritis and the associated glomerular injury. Treatment with
EET-B resulted in significant reductions of both nephrinuria and glomerular injury in Dahl SS rats (Figure 3A–3C). The kidney protective effect of the EET analog EET-B was further evident with its effect on the plasma creatinine and blood urea nitrogen (BUN) levels. There was marked increase in plasma creatinine (3.0±0.6 versus 1.5±0.2 mg/dL; \( P<0.05 \)) and BUN (53.4±6.0 versus 36.6±2.0 mg/dL; \( P<0.05 \)) levels in Dahl SS rats compared with that in SSBN13 rats. EET-B treatment reduced both plasma creatinine (1.7±0.2 mg/dL) and BUN (32.0±2.0 mg/dL) levels in Dahl SS rats (\( P<0.05 \)).

**EET-B Treatment Reduced Renal Inflammation in Dahl SS Rats**

Marked inflammation was observed in Dahl SS rats receiving high-salt diet. In relation to this, Dahl SS rats demonstrate 70% greater urinary excretion of MCP-1 compared with SSBN13 rats. In parallel to elevated urinary MCP-1, Dahl SS rats also demonstrated 50% to 65% greater infiltration of macrophages/monocytes in the kidney of these rats compared with that in SSBN13 rats. Treatment with EET-B reduced inflammation in Dahl SS rats with marked reduction in urinary MCP-1 (50%) and infiltration of macrophages in the renal cortex (40%) and medulla (35%) of these rats (Figure 4A–4D). In the present study, kidney T-lymphocyte infiltration was determined, and there was a 30% increase in T-lymphocyte infiltration in the kidney cortex of Dahl SS rats, which was not significantly reduced by EET-B treatment (Figure S3).

**EET-B Treatment Reduced Renal and Cardiac Fibrosis in Dahl SS Rats**

In this present study, we have also observed renal fibrosis in Dahl SS rats involving 5% and 14% of the kidney cortical and medullary regions, respectively. EET-B treatment reduced renal fibrosis by 40% to 50% in cortical and medullary regions of the kidney of Dahl SS rats (Figure 5A–5C). In relation to renal fibrosis, we also observed marked elevation in the mRNA expression of tumor growth factor-\( \beta \) in the kidney of Dahl SS rats, and EET-B treatment reduced this expression (Figure S4). In addition to renal fibrosis, Dahl SS rats had increased cardiac fibrosis compared with SSBN13 rats, and EET-B treatment markedly attenuated this cardiac fibrosis in Dahl SS rats (Figure S5).

**EET-B Treatment Reduced Renal Oxidative Stress in Dahl SS Rats**

Dahl SS rats receiving high-salt diet exhibited marked oxidative stress with 40% to 60% elevated levels of malondialdehyde (MDA) in cortex and medulla of the kidney compared with that of SSBN13 rats. EET-B treatment attenuated oxidative stress in Dahl SS rats with marked reductions of renal cortical and medullary contents of MDA by 25% to 35% (Figure 6A and 6B). The antioxidative effects of EET-B in Dahl SS rats were further observed in terms of a significant 42% reduction in urinary 8-isoprostane excretion, which was markedly elevated in Dahl SS rats compared with SSBN13 rats (Figure 6C). Dahl SS rats exhibited marked reduction in renal excretion of nitrate and nitrite, NO metabolites, compared with SSBN13 rats. EET-B treatment increased renal excretion of NO metabolites in Dahl SS rats by 40% and further indicated antioxidant effect of EET-B in Dahl SS rats (Figure 6D).

**EET-B Treatment Reduced Renal ER Stress in Dahl SS Rats**

Apart from renal oxidative stress and inflammation, Dahl SS rats also exhibited marked ER stress with elevated mRNA expression of ER stress markers, GRP 78 and CHOP, in the kidney. We found a 4.5-fold greater expression of GRP 78 and a 3.5-fold greater expression of CHOP mRNA in the kidney of Dahl SS rats compared with that in SSBN13 rats (Figure 7A and 7B). In line with these observations on the renal mRNA expression

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**Figure 3.** A. Urinary excretion of nephrin. B. Representative photomicrographs of periodic acid-Schiff staining (\( \times200 \)) depicting glomerular injury with mesangial expansion (yellow arrows) and other changes related to glomerular sclerosis in different experimental groups. C. Semiquantitative scoring of glomerular injury in rats from different experimental groups. \( \ast P<0.05 \) vs SSBN13 rats treated with vehicle; \( \#P<0.05 \) vs vehicle-treated Dahl salt-sensitive (Dahl SS) rats. Data expressed as mean±SEM, \( n=6 \) to 7. EET-B indicates epoxyeicosatrienoic acid analog B; and HS, high-salt diet.
of GRP 78 and CHOP, we also found marked elevation in the immunoreactivity positive levels for GRP 78 and CHOP in the kidney of Dahl SS rats compared with that in SSBN13 rats (Figure 7C). Interestingly, treatment with EET-B markedly reduced renal ER stress in Dahl SS rats that was evident from a 70% to 72% reduction in the renal mRNA expressions of GRP 78 and CHOP along with reduced immunoreactivity of these ER stress markers in the kidney (Figure 7A–7C).

Discussion

There is strong evidence that EETs have the ability to protect organs by mechanisms involving anti-inflammatory and antioxidative activities. Indeed, it has been demonstrated that EETs provide organ protection in a number of preclinical rodent models of human diseases, including hypertension, diabetes mellitus, and ischemic cardiac injury.9–11,25,27,28 One approach to target EET for combating diseases is the development of EET analogs that are designed to resist metabolism and have improved bioavailability.15,16 Recently, we developed a novel orally active EET analog, EET-B, and determined its EET analog property. We clearly demonstrated that EET-B is an EET analog because the EET antagonist 14,15-EEZE abolished EET-B–mediated mesenteric resistance artery vasodilatation and the anti-inflammatory activity of EET-B in cultured endothelial cells.

Figure 4. Urinary excretion of monocyte chemoattractant protein-1 (MCP-1; A), mean ED-1 or CD68 positive macrophage/monocyte counts in the kidney cortex (B) and medulla (C) of rats from different experimental groups. Representative photomicrographs of immunohistochemical staining depicting ED-1 or CD68 positive macrophage/monocyte (yellow arrows) in the kidney of rat from different experimental groups (D). *P<0.05 vs SSBN13 rats treated with vehicle; #P<0.05 vs vehicle-treated Dahl salt-sensitive (Dahl SS) rats. Data expressed as mean±SEM, n=6 to 7. EET-B indicates epoxyeicosatrienoic acid analog B; and HS, high-salt diet.

Figure 5. Representative photomicrographs of Masson’s Trichrome staining of kidney cortical (A) and medullary (B) sections depicting fibrosis (yellow arrows) along with the calculated fibrotic area (%) in the renal cortical and medullary sections of rats from different experimental groups (C). *P<0.05 vs SSBN13 rats treated with vehicle; #P<0.05 vs vehicle-treated Dahl salt-sensitive (Dahl SS) rats. Data expressed as mean±SEM, n=6 to 7. EET-B indicates epoxyeicosatrienoic acid analog B; and HS, high-salt diet.
In several earlier studies, EET analogs have demonstrated organ protection in a number of experimental disease models. It is known that salt-sensitive hypertension in human and experimental animal models has been associated with progressive kidney damage leading to end-stage renal disease caused by elevated inflammation and oxidative stress. Consequently, in the present study, we hypothesized that the novel orally active EET analog, EET-B, with its strong organ protective activities, ameliorates salt-sensitive hypertension and the associated kidney injury. We used Dahl SS rats that serve as an excellent model of salt-sensitive hypertension and the associated kidney injury, exhibiting many phenotypic characteristics common in human hypertension.

We have demonstrated that EET-B provided strong kidney protection in salt-sensitive Dahl SS rats, evidenced by reductions of a number of important and relevant kidney injury markers. The renal protection is further evident from histopathologic observations including markedly reduced tubular cast formation and fibrosis in the kidney. In relation to our findings in the present study, we have earlier demonstrated organ protective effects by pharmacological and genetic manipulation of EET metabolic pathway in different pathologies including hypertension. Pharmacological inhibition or genetic knockout of sEH enzyme (sEHi and Ephx2 KO) that catalyzes the conversion of EETs to their diols have consistently been demonstrated to decrease kidney and heart damage associated with hypertension and other cardiovascular diseases. One limitation of sEH inhibitors is that they result in a generalized increase in EETs, and their biological effects depend on

Figure 6. Renal cortical (A) and medullary (B) contents of malondialdehyde (MDA) in rats of different experimental groups. Urinary excretion of 8-isoprostane (C) and nitrate/nitrite (D) in rats from different experimental groups. *P<0.05 vs SSBN13 rats treated with vehicle; #P<0.05 vs vehicle-treated Dahl salt-sensitive (Dahl SS) rats. Data expressed as mean±SEM, n=6 to 7. EET-B indicates epoxyeicosatrienoic acid analog B; and HS, high-salt diet.

Figure 7. Renal mRNA expression of endoplasmic reticulum stress markers glucose regulatory protein 78 (GRP78; A) and CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP; B) in the kidney of rats from different experimental groups. Representative photomicrographs of immunohistochemical staining showing the expression and localization (black arrows) of GRP78 and CHOP in the kidney of rats from different experimental groups (C). *P<0.05 vs SSBN13 rats treated with vehicle; #P<0.05 vs vehicle-treated Dahl salt-sensitive (Dahl SS) rats. Data expressed as mean±SEM, n=6 to 7. EET-B indicates epoxyeicosatrienoic acid analog B; and HS, high-salt diet.
the generation of EETs by CYP epoxygenase enzymes. It is further known that in pathological conditions, including that of Dahl SS rat, the endogenous EET generation can be low because of decreased levels or activities of CYP epoxygenase enzymes.\textsuperscript{8,34,35} The experimental findings of the present set of experiments provide evidence that an EET analog decreases kidney injury in Dahl SS hypertension.

In the present study, we demonstrated that EET-B provided kidney protection in Dahl SS rats, and that this protection was independent of any change in blood pressure and sodium excretion. Indeed, it is known that the Dahl SS rat is a genetic model of salt-dependent hypertension that exhibits impaired sodium excretion, the hallmark of salt-sensitive hypertension. It is also known that a mechanism by which EETs lower blood pressure is via a natriuretic effect.\textsuperscript{35} It is important to note that the structure of EET-B is designed to mimic 14,15-EET, and that 14,15-EET has been demonstrated to have variable effects on epithelial sodium transport, which is pivotal for EETs natriuretic effect.\textsuperscript{36–38} In relation to this, in the present study, we demonstrate that EET-B does not affect renal sodium excretion in the Dahl SS rat and does not inhibit sodium transport in isolated mpkCCD\textsubscript{14} cells. Thus, despite the vasodilator, antioxidative, and anti-inflammatory effects of EET-B, the lack of an EET-B effect on sodium transport could explain the inability of EET-B to lower blood pressure in Dahl SS rat.

Similar to our findings in the present study, inhibitors of sEH, an important modulator of CYP epoxygenase pathway, also demonstrated blood pressure-independent kidney protective actions. For example, experimental studies in type 2 diabetic rats receiving high-salt diet along with angiotensin II hypertension demonstrated that sEH inhibition could provide renal protection without lowering blood pressure.\textsuperscript{13} Similarly, it is highly plausible that the kidney protective effect of EET-B is related to properties other than effects on blood pressure. Indeed, our earlier experimental studies indicated that increased EET bioavailability by sEH inhibition possesses strong anti-inflammatory and antioxidative effects associated with its organ protective effects. For instance, pharmacological inhibition of sEH in hypertensive Goto-Kakizaki rats and disruption of sEH gene, Ephx2, in mice attenuated progression of kidney damage associated with hypertension and diabetes mellitus.\textsuperscript{13,14} These studies further demonstrated that the kidney protective effect of sEH inhibition by pharmacological and genetic manipulation was strongly associated with antioxidative and anti-inflammatory effects of EETs.\textsuperscript{12–14} With this background, it is possible that the observed EET-B–mediated kidney protection in Dahl SS rats is associated with antioxidative and anti-inflammatory properties of this orally active novel EET analog.

Indeed, kidney injury in Dahl SS rat is strongly associated with inflammation and oxidative stress. A number of studies demonstrated enhanced oxidative stress with elevated production of vascular superoxide and increased plasma H\textsubscript{2}O\textsubscript{2} concentration in hypertensive Dahl SS rats.\textsuperscript{27,39–41} It is widely demonstrated that the renal damage frequently observed in Dahl SS rat is related to increased oxidative stress involving reduced NO bioavailability and increased superoxide production in the kidney.\textsuperscript{39–43} A number of studies demonstrated that oxidative stress plays an important role in the development and progression of renal injury associated with excessive dietary salt ingestion.\textsuperscript{29,30,44} In line with these observations, we have observed marked oxidative stress with elevated kidney MDA levels, increased urinary excretion of 8-isoprostone, and reduced urinary excretion of nitrate/nitrite in Dahl SS rats. Interestingly, EET-B treatment reduced oxidative stress in these rats by reducing kidney MDA level, urinary excretion of 8-isoprostone, and by increasing urinary excretion of nitrate/nitrite. In support of our findings, in a recent study, it was reported that upregulation of epoxygenases and increased EETs increased the expression and activity of antioxidant enzyme superoxide dismutase during toxic insult, and consequently reduced oxidative stress.\textsuperscript{45} Another recent study demonstrated that renal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and urinary MDA excretion were reduced in diabetic rats with genetically disrupted sEH system, and suggested the kidney protective role of EETs in diabetic renal pathology in relation to their antioxidant effect.\textsuperscript{14} Thus, the findings of the current study and previous studies provide convincing evidence that antioxidant actions in response to increasing EETs contribute to their organ protection.

Apart from antioxidative effect, the kidney protective potential of EETs is also associated with anti-inflammatory activity.\textsuperscript{13,14,45–48} These earlier reported findings in different pathologies indicated a strong possibility that the kidney protective effect of EET-B in Dahl SS rats could be attributable to a reduction in inflammation. As reported in earlier studies,\textsuperscript{47,48} indeed, we have observed renal inflammation in Dahl SS rats. There was marked elevation in the urinary MCP-1, kidney macrophage/monocyte, and T-lymphocyte infiltration in Dahl SS rat. EET-B treatment significantly decreased MCP-1 and kidney macrophage/monocyte but not T-lymphocyte infiltration in Dahl SS rats. Our observations support several earlier reports on the anti-inflammatory activity of EETs that has been implicated in protecting kidney in a number of pathologies. Increased bioavailability of EETs by genetic disruption of sEH reduces inflammation and provides kidney protection in streptozotocin-induced diabetes mellitus and in salt-sensitive hypertension.\textsuperscript{12,14} Therefore, in the present study, we clearly demonstrated that along with marked reduction in oxidative stress, an attenuation of renal inflammatory response contributed to the EET analog EET-B–mediated kidney protection in Dahl SS rat.

Many reports indicated that ER stress and inflammation are linked and contribute to the pathophysiology of chronic inflammatory diseases such as diabetes mellitus and hypertension. Indeed, evidence suggests that ER stress is a potential mediator of inflammation in cardiovascular diseases.\textsuperscript{49} In a recent study, ER stress was implicated in cardiac injury associated with angiotensin II hypertension in mice. Angiotensin II hypertension caused ER stress that was associated with cardiac hypertrophy and fibrosis in mice, and these pathological events were ameliorated by pharmacological inhibition of ER stress.\textsuperscript{50} Consequently, in the present study, we have investigated the involvement of ER stress and demonstrated marked elevation in renal mRNA expression of ER stress markers GRP 78 and CHOP in Dahl SS rat. Interestingly, EET-B treatment markedly attenuated expression of these ER stress markers in the kidney of Dahl SS rats as evident from reduced mRNA expression.
and immunoreactivity positive for GRP 78 and CHOP. These observations indicate involvement of ER stress in the kidney injury of Dahl SS rat and, most importantly, indicate a novel mechanism by which EET could protect kidney in a pathological condition related to salt-sensitive hypertension.

Perspective

We have clearly demonstrated the potential of a novel orally active EET analog in ameliorating renal damage in salt-sensitive hypertension. We provide substantial evidence that this novel EET analog ameliorated kidney injury by reducing renal inflammation, oxidative stress, and ER stress in Dahl SS hypertension. Importantly, this study established the compelling potential of EET-based therapeutics to treat progressive kidney injury in salt-sensitive hypertension. Moreover, with antioxidative, anti-inflammatory, and anti-ER stress, the novel orally active EET analog, EET-B, holds therapeutic promise for a number of cardiovascular pathologies that lead to end-organ damage.

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Disclosures

None.

References


What Is New?

This study demonstrated therapeutic promise of a novel orally active epoxyeicosatrienoic acid (EET) analog in hypertensive kidney diseases.

What Is Relevant?

In this study, we unveil kidney protective mechanism of a novel orally active EET analog in hypertensive kidney diseases.

We demonstrated that this EET analog provided kidney protection in salt-sensitive hypertension by reducing inflammation, oxidative stress, and endoplasmic reticulum stress in the kidney.

Summary

Salt-sensitive hypertension leads to end-organ damage that is associated with elevated oxidative stress, inflammation, and endoplasmic reticulum stress. We have demonstrated promising kidney protective effect of a novel EET analog in a preclinical model of human salt-sensitive hypertension. We have clearly demonstrated that EET analog ameliorated kidney injury by reducing renal oxidative stress, inflammation, and endoplasmic reticulum stress.
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AN ORALLY ACTIVE EPOXYEICOSATRIENOIC ACID ANALOG ATTENUATES KIDNEY INJURY IN HYPERTENSIVE DAHL SALT SENSITIVE RAT

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Extended Materials and Methods

**Vascular reactivity study**

Second order mesenteric artery segments were mounted between two glass cannulas in a pressure myograph system (Danish Myo Technology model 111P, DMT, Aarhus, Denmark). The vessel was oxygenated in 95% O₂/5% CO₂ Krebs physiological salt solution at pH 7.4 and 37°C. Under no-flow conditions, the pressure within the vessel was increased in 10-mmHg increments from 20 to 65 mmHg. The vessel was then equilibrated at 65 mmHg for 30 min in the presence of the nitric oxide synthase (NOS) inhibitor, Nω-nitro-L-arginine-methyl ester (L-NAME, 100µM) and the cyclooxygenase inhibitor indomethacin (10µM). Pressure was maintained at 65 mmHg for the duration of the experiment. Lumen diameter measurements were acquired using the MyoView 1.2P user interface (DMT). Control lumen diameter was calculated as the mean diameter during the last minute of the 30-min equilibration. Vessels were constricted with the thromboxane mimetic U-46619 and diameter of the constricted vessel was calculated as the mean during the last minute of the 15 minute period. EET antagonist 14,15-EEZE (10µM) or vehicle was added to the bath solution for 10 minutes prior to addition of EET-B. Graded concentrations of EET-B (10⁻⁹ to 10⁻⁵M) were added to the bath solution every 5 minutes, and lumen diameter to each EET-B concentration was measured. The nitric oxide donor, sodium nitroprusside (100µM) was added to the bath at the end of the experimental period to ensure the vascular integrity.

**Biochemical analysis**

Urinary biochemical analysis was done using commercially available colorimetric, ELISA and EIA assay kits; creatinine, 8-isoprostane and nitrate/nitrite from Cayman Chemical (Ann Arbor, MI, USA), albumin and nephrin from Exocell (Philadelphia, PA, USA), and monocyte chemoattractant protein-1 (MCP-1) from BD Biosciences (San Jose, CA, USA). The levels of blood urea nitrogen (BUN) (BioAssay Systems, Hayward, CA, USA) and plasma creatinine (Cayman Chemical) were measured spectrophotometrically using commercial kits. Kidney tissue malondialdehyde (MDA) was measured in the kidney using a commercially available kit (Cayman Chemical). To determine the kidney tissue MDA level, the rat kidney was homogenized with buffer containing 1.5% potassium chloride to obtain a 1:10 (w/v) whole kidney homogenate. MDA was measured spectrophotometrically after reaction with thiobarbituric acid.
**Kidney histology**
For histological analysis, kidneys were excised, longitudinally sectioned, immersion-fixed in 10% neutral buffered formalin and paraffin embedded. The kidney sections were embedded and cut into 4 µm slices for use in histology protocols. Formalin-fixed paraffin-embedded kidney slices were deparaffinized, re-hydrated and stained with Masson’s Trichrome Periodic Acid-Schiff (PAS) reagents. Glomerulosclerosis and mesangial matrix expansion were blindly scored from kidney sections stained by PAS reagent at a magnification of 400x to determine the glomerular damage using the following numeric scale: 0= no damage; +1= very mild; +2= mild; +3= moderate and +4= severe. Two observers in a blinded fashion conducted histological analysis of glomerular injury. Tubules containing proteinaceous casts were determined in the PAS stained slides at magnification of 200x using Nikon NIS Elements Software. The percentage area positive for cast was calculated from the mean of 8 cortical and 5 medullary fields (200x) for each animal. Renal fibrosis was assessed in Masson’s Trichrome stained slides and the percentage of the total area positive for interstitial collagen was calculated as an index of fibrosis using Nikon NIS Elements Software. The percentage area positive for interstitial collagen was calculated from the mean of 8 cortical and 5 medullary fields (200x) for each animal. Two observers who were blind to the treatment protocol and the animal models carried out the renal cast and fibrosis area calculations. Cardiac fibrosis was assessed from interstitial collagen positive areas in Picro-sirius Red stained tissue slides using Nikon NIS Elements Software.

**Immunohistochemical analysis**
The kidney sections were embedded and cut into 4 µm slices for use in immunohistochemistry protocols. Formalin-fixed paraffin-embedded kidney slices were deparaffinized, re-hydrated and subjected to immunohistochemistry protocols. Kidney sections were immunostained with anti-CD68 (1:100; Serotec, Raleigh, NC, USA) and anti-CD43 to determine macrophage/monocyte infiltration and T-lymphocytes, respectively. Biotinylated rat anti-mouse secondary antibody (1:200) was used for development with avidin-biotinylated HRP complex (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) followed by counterstaining with hematoxylin and mounted for image capturing. Stained sections were visualized by light microscopy at 400x magnification and digital images of the stained kidney were taken for analysis. Macrophage/monocyte infiltration was determined by point counting of CD68-positive cells by an experienced reviewer blinded to the experimental groups. The number of positive cells per picture was divided by the metric area of the image, established by micrometer slide image to obtain the number of positive cells per mm$^2$. Infiltration of T-lymphocytes in the kidney was determined as the percentage area positive for CD43 (1:200; Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA, USA) immunostaining was assessed at a magnification of 200x using Nikon NIS Elements Software. Additional kidney sections were also immunostained with antibodies against ER stress markers, glucose regulatory protein 78 (GRP78, 1:100; Santa Cruz Biotechnology, USA) and C/EBP homologous protein (CHOP, 1:200, Santa Cruz Biotechnology) in order to determine the relative expression level and localization of these ER stress markers in the kidney. Biotinylated horse anti-goat or anti-rabbit secondary antibodies (1:200, Santa Cruz Biotechnology) were used for development with avidin-biotinylated HRP complex (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA). Finally the slides were counterstained with hematoxylin and mounted for image capturing.

**Real-Time PCR Analyses**
Total RNA was isolated from kidney homogenate using TRIzol LS reagents (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. The isolated RNA was treated with RNase-free DNase (Invitrogen Life Technologies, Carlsbad, CA, USA) to remove traces of genomic DNA contamination. The mRNA samples were quantified by spectrophotometry at 260 nm and
1 µg of total RNA was reverse-transcribed to cDNA using iScript™ Select cDNA Synthesis Kit (Bio-
Rad, Hercules, CA, USA). The target gene expression was quantified by iScript One-Step RT-PCR Kit
with SYBR green using MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories,
Hercules, CA, USA). Each amplified sample in all wells was analyzed for homogeneity using dissociation
curve analysis using iQ5 Optical System Software, Version 2.1 (Bio-Rad Laboratories, Hercules, CA,
USA). After denaturation at 95°C for 2 min, 40 cycles were performed at 95°C for 10 s and at 60°C for
30s. Each sample was run in triplicate, and the comparative threshold cycle (Ct) method was used to
quantify fold increase (2^{-ΔΔCt}) in the expression of the target genes compared to controls. In analyzing
the relative expression of the target genes, the Ct values were normalized to a housekeeping gene (pgk1).
Statistical analyses were carried out for at least 5-7 experimental samples in each experimental group.

**Endothelial Cell Culture Studies**

Human umbilical vein endothelial cells (HUVECs) grown in 6-well plates were pretreated with vehicle
(n=4), EET-B (5µM, n=4), and a combination (n=4) of EET-B (5µM) and EET antagonist 14,15-EEZE
(5µM) for 16h. All samples were exposed to TNF-α (10ng/ml) for 6h at 37°C and the culture medium was
assayed for the measurement of monocyte chemoattractant protein-1 (MCP-1) (Thermo Fisher Scientific,
Rockford, IL, USA).

**Na+ Transport Studies**

Immortalized mouse cortical collecting duct (mpkCCDc14) principal cells were grown in defined medium
on permeable supports (Costar Transwells, 0.4-µm pore, 24-mm diameter) as described previously.1,2 A
Millicel Electrical Resistance System (Millipore, Billerica, MA) was used to measure voltage and
resistance across the mpkCCDc14 cell monolayers grown on permeable supports as described
previously.2,3 Equivalent transepithelial Na⁺ currents were calculated as the quotient of transepithelial
voltage to transepithelial resistance under short-circuit conditions.

**Determination of plasma levels of endogenous EETs and DHETs**

Plasma levels of endogenous EETs and DHETs were measured by LC–ESI–MS (Agilent 6460 Triple
Quad LC/MS). Samples for chromatographic analysis were prepared from 200µl of plasma using solid
phase extraction with Varian Bond Elut® C18 column (Agilent Technologies, Santa Clara, CA, USA).
In the LC–ESI–MS analysis process, the samples (5µl) were separated on a reverse phase C18 column
(Kromasil, 250 x2 mm) using water/acetonitrile containing 0.005% acetic acid as a mobile phase at the
flow rate of 0.20 mL/min. The mobile phase started at 90% methanol for 2 min, linearly increased to
100% methanol in 10 min, and held for 10 min. Drying gas flow was 12 l/min, drying gas temperature
was 350°C, nebulizer pressure was 35 psig, vaporizer temperature was 325°C, capillary voltage was 3000
V, and fragmentor voltage was 120 V.
References


Figure S1: Human umbilical vein endothelial cells (HUVEC) were pretreated with vehicle, EET-B (5μM) or a combination of EET-B (5μM) and EET antagonist 14,15-EEZE (5μM) for 16h followed by 6h incubation with TNF-α (10ng/ml). TNF-α increased monocyte chemoattractant protein-1 (MCP-1) levels (*p<0.05 vs. vehicle), and EET-B attenuated TNF-α mediated increase in MCP-1 levels (#p<0.05 vs. TNF-α). 14,15-EEZE abolished the anti-inflammatory effect of EET-B (†p<0.05 vs EET-B). Data expressed as mean ± SEM, n=4.
Figure S2: Effect of EET-B on basal Na$^+$ ion transport in mpkCCDc14 cells. The figure is demonstrating the equivalent short circuit current (Isc) in mpkCCDc14 principal cells in response to EET-B administration. EET-B did not alter the transepithelial Na$^+$ current in mpkCCDc14 cells. Data expressed as mean $\pm$ SEM, n=4.
Figure S3: Percentage (%) of CD43 immunopositive kidney areas showing the presence of T-lymphocytes in the kidney cortex (A) and medulla (B) of rats from different experimental groups. Data expressed as mean ± SEM, n=4.*p<0.05 vs. SSBN13.
Figure S4: Renal mRNA expression of tumor growth factor-β (TGF-β) in the kidney cortex (A) and medulla (B) of rats from different experimental groups. *p<0.05 vs. SSBN13 rats treated with vehicle; #p<0.05 vs. vehicle treated Dahl SS rats. Data expressed as mean ± SEM, n=6-7.
Figure S5: Representative photomicrographs of Picro-sirius Red staining of heart tissues depicting collagen positive areas (white arrows) (A) along with the calculated collagen positive area (%) in the heart sections of rats from different experimental groups (B). *p<0.05 vs. SSBN13 rats treated with vehicle; #p<0.05 vs. vehicle treated Dahl SS rats. Data expressed as mean ± SEM, n=4.
Figure S6: Plasma EET to DHET ratio in Dahl SS rats treated with vehicle or EET-B. Plasma EETs and DHETs were measured using LC-ESI-MS and all four regioisomers of EET were detected. EET levels averaged 19±2 and 5.2±0.4 ng/ml in vehicle or EET-B treated Dahl SS rats; respectively. DHET levels averaged 13±2 and 2.1±0.1ng/ml in vehicle or EET-B treated Dahl SS rats; respectively. Data expressed as mean ± SEM, n=4.