Role of haem oxygenase in the renoprotective effects of soluble epoxide hydrolase inhibition in diabetic spontaneously hypertensive rats

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Abstract

We have shown previously that inhibition of sEH (soluble epoxide hydrolase) increased EETs (epoxyeicosatrienoic acids) levels and reduced renal injury in diabetic mice and these changes were associated with induction of HO (haem oxygenase)-1. The present study determines whether the inhibition of HO negates the renoprotective effect of sEH inhibition in diabetic SHR (spontaneously hypertensive rats). After 6 weeks of induction of diabetes with streptozotocin, SHR were divided into the following groups: untreated, treated with the sEH inhibitor t-AUCB (trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid), treated with the HO inhibitor SnMP (stannous mesoporphyrin), and treated with both inhibitors for 4 more weeks; non-diabetic SHR served as a control group. Induction of diabetes significantly increased renal sEH expression and decreased the renal EETs/DHETEs (dihydroxyeicosatrienoic acid) ratio without affecting HO-1 activity or expression in SHR. Inhibition of sEH with t-AUCB increased the renal EETs/DHETEs ratio and HO-1 activity in diabetic SHR; however, it did not significantly alter systolic blood pressure. Treatment of diabetic SHR with t-AUCB significantly reduced the elevation in urinary albumin and nephrin excretion, whereas co-administration of the HO inhibitor SnMP with t-AUCB prevented these changes. Immunohistochemical analysis revealed elevations in renal fibrosis as indicated by increased renal TGF-β levels and fibronectin expression in diabetic SHR and these changes were reduced with sEH inhibition. Co-administration of SnMP with t-AUCB prevented its ability to reduce renal fibrosis in diabetic SHR. In addition, SnMP treatment also prevented t-AUCB-induced decreases in renal macrophage infiltration, IL-17 expression and MCP-1 levels in diabetic SHR. These findings suggest that HO-1 induction is involved in the protective effect of sEH inhibition against diabetic renal injury.

Key words: albuminuria, diabetic renal injury, haem oxygenase, inflammation, soluble epoxide hydrolase (sEH)

INTRODUCTION

Diabetic renal injury is one of the major complications of diabetes and often leads to end-stage renal disease with the disease progression [1,2]. Diabetic renal injury is often characterized by glomerulosclerosis, albuminuria, hypertension and a decline in GFR (glomerular filtration rate) [1–3]. Many factors contribute to the progression of diabetic renal injury such as poor glycemic control and its associated hyper-filtration and increased glomerular capillary pressure, hypertension, obesity, lack of physical activity and daily stress [3,4]. The pathophysiology of diabetic renal injury is multifactorial; however, inflammation and oxidative stress are the main keys in the pathogenesis of the disease [5]. Because induction of diabetes with streptozotocin increases proteinuria and oxidative stress without a change in arterial pressure in Sprague–Dawley rats [6], superimposing streptozotocin-induced diabetes on hypertension background could provide a better clinically relevant picture to signs and symptoms of diabetic renal injury. Accordingly, we have recently demonstrated that diabetic SHR (spontaneously hypertensive rats) are an
excellent experimental model of diabetic renal injury, where diabetes coexists with hypertension and together exacerbate the progression of renal inflammation and injury similar to the clinical manifestations of the disease [7].

The CYP (cytochrome P450) mono-oxygenase pathway catalyses the oxidation of arachidonic acid at any of the four double bonds to four regioisomeric EETs (epoxyeicosatrienoic acids) (5,6-, 8,9-, 11,12- and 14,15-EET) by the CYP epoxygenase [5,8]. EETs are EDHFs (endothelium-derived hyperpolarizing factors) with anti-hypertensive and anti-inflammatory properties; however, conversion of EET epoxides into their corresponding diols (DHETs) by sEH (soluble epoxide hydrolase) enzyme limits EETs availability and decreases their potential use to combat cardiovascular disease [5,8]. Accordingly, inhibition of sEH has emerged as a promising approach in increasing EET levels, and consequently provides protection against cardiovascular disease including diabetic renal injury [9]. Recently, we have demonstrated that induction of HO (haem oxygenase)-1 reduces renal inflammation and injury in diabetic SHR via anti-oxidant and anti-inflammatory mechanisms [7]. We have also shown that increased EET levels induce HO-1 in diabetic mice, which protects the kidney from diabetes-induced renal injury via the reduction in renal inflammation and oxidative stress [9]. Accordingly, the present study was designed to test the hypothesis that inhibition of HO will negate the renoprotective effects of sEH inhibition in diabetic SHR in which the coexistence of hypertension and diabetes exacerbates renal damage. Furthermore, most of the previous studies test the efficacy of sEH inhibitors as a preventive treatment for end-organ damage in cardiovascular disease; however, our study was designed to test the efficacy of sEH inhibitors as an interventional treatment for diabetic renal injury. Thus diabetic SHR were treated with an sEH inhibitor after 6 weeks of induction of diabetes when the clinical features of diabetic renal injury already exist [7] to determine whether inhibition of sEH will prevent or slow the progression of diabetic renal injury.

MATERIALS AND METHODS

Animals

All procedures with animals were performed in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals and Georgia Regents University guidelines. Ten-week-old male SHR (Charles River) were used in this study. Diabetes was induced by a single injection of streptozotocin (Sigma; 60 mg/kg of body mass, intravenously) and rats were supplemented with sustained release insulin implants (subcutaneous; Lanshin) to mimic uncontrolled diabetes by maintaining blood glucose levels within the 500–550 mg/dl range. Blood glucose was tested weekly using a glucometer. After 6 weeks of induction of diabetes, SHR were divided into the following groups: untreated, treated with the sEH inhibitor t-AUCB (trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyl]oxy]benzoic acid) (10 mg/l in drinking water) as a selective inhibitor to the hydrolase domain of the sEH, treated with the HO inhibitor SnMP (stannous mesoporphyrin) (10 mg/100 g of body mass, intraperitoneal weekly), and treated with both inhibitors for 4 weeks; non-diabetic SHR served as a control group (n = 6–8/group). SBP (systolic blood pressure) was measured weekly using the tail-cuff method (ITC Life Science). At the end of the 4 weeks treatment, rats were placed in metabolic cages (Nalgene) for 24 h urine collection. Rats were anaesthetized with sodium pentobarbital (50 mg/kg of body mass) and blood samples were collected from the abdominal aorta for the flow cytometry assessment of IL-17 (interleukin-17). The kidneys were removed and one kidney was used to isolate glomeruli and the other was used to isolate cortex. Urinary albumin and nephrin excretion levels (Exocell) were determined as indices of renal injury. Urinary 8-OHdG (8-hydroxy deoxyguanosine) excretion levels were assessed as marker of oxidative stress. Urinary and plasma creatinine levels were assessed using picric acid method as described previously [6] and were used to calculate creatinine clearance.

Isolation of glomeruli

Glomeruli were isolated from the kidney by a gradual sieving technique as described previously [7] and were used to determine glomerular active TGF-β1 (transforming growth factor β-1) concentrations using the Quantikine TGF-β1 kit (R&D Systems).

Renal EETs/DHETEs (dihydroxyeicosatrienoic acids), NF-κB (nuclear factor κB), MCP-1 (monocyte chemotactic protein-1) and HO-1 assessment

The LC (liquid chromatography)/MS method was used to measure the ratio of renal EETs/DHETEs in the kidney cortex as described previously [9]. A nuclear extract kit from Cayman Chemicals was used to prepare nuclear extract from the kidney cortex. The nuclear extract was used for the determination of NF-κB activity using the TransAM p65-NF-κB transcription factor assay kit (Cayman Chemicals) and the absorbance was normalized per ng of nuclear extract protein. Cortical MCP-1 (BD Biosciences) was measured as an index of inflammation. Cortical HO-1 activity was also assessed using commercially available kit from Enzo Life Sciences.

Homogenization of renal cortex for protein expression

Kidney cortex was homogenized in ice-cold homogenization buffer in the presence of protease inhibitors and used for determination of sEH, HO-1 and HO-2 protein expression using Western blotting as described previously [6]. The primary antibodies were purchased from Santa Cruz Biotechnology. These antibodies were detected with a horseradish peroxidase-conjugated secondary antibody and ECL chemiluminescence (Amersham BioSciences). Intensity of immunoreactivity was measured by densitometry and β-actin was used to verify equal protein loading.

Renal histopathology

In a separate set of rats (n = 3–4/group), kidneys were perfused with 10% (v/v) formalin solution and we then paraffin embedded and cut into 5 μm sections. Kidney sections were used for immunohistochemical evaluation of nephrin as a marker of glomerular injury, fibronectin as a marker of renal fibrosis, F4/80 as a marker for activated macrophage infiltration, and IL-17 as a
marker of inflammation (antibodies from Santa Cruz Biotechnology). Kidney sections from different rat groups were also stained with Masson’s trichrome staining to assess the amount of collagen deposition. Ten microscopic images of the kidney cortex per rat were randomly taken at ×200 magnification. For Masson’s trichrome staining intensity assessment, scoring of the slides was performed blindly and graded on a scale of 1–10. For immunohistochemistry assessment, intensity of renal tissue staining was performed using Metamorph image analysis software. Numerical values of immunoreactive staining intensity in each image were assessed and this was followed by calculation of the percentage ratio of immunoreactive to non-reactive areas. Using a double labelling immunofluorescence technique, additional kidney sections from rat groups were co-stained with anti-CD3 (specific for T-cells) and anti-IL-17 antibodies to determine whether T-cells are the cellular sources of IL-17. A total of ten microscopic images of kidney cortex per rat were randomly taken at ×400 magnification.

**Analytical flow cytometry**

Blood samples were incubated with fluorochrome-conjugated surface marker for Pan T-cells (anti CD3 antibody; BD Biosciences) on ice for 20 min in the dark (n = 4 per group). Following one wash with PBS, samples were permeabilized for intracellular staining of IL-17 using BD Cytofix/Cytoperm buffer (BD Biosciences) for 15 min. Samples were incubated with anti-IL-17 antibody (BD Biosciences) for 20 min in the dark on ice. Samples were washed once with PBS and flow cytometric analyses were performed on samples using FACSCalibur machine (BD Biosciences) equipped with 488 nm argon (for FITC, PE, PE-CY5) and 647 nm krypton (for allophycocyanin) lasers. Cells were gated based on forward and side scatter properties and on marker combinations to select cells of interest.

**Statistical analysis**

All results are means ± S.E.M. Data were analysed using ANOVA followed by Tukey’s post-hoc test for multiple group comparisons. Differences were considered statistically significant with P < 0.05 compared with the control. Analyses were performed using GraphPad Prism version 4.0 software.

**RESULTS**

Induction of diabetes with streptozotocin did not exacerbate the elevation in blood pressure in SHR (SBP was 178 ± 5 mmHg in diabetic SHR compared with 175 ± 3 mmHg in control SHR). Treatment of diabetic SHR with the sEH inhibitor t-AUCB did not lower SBP (178 ± 2 mmHg). Inhibition of HO with SnMP also did not significantly exacerbate the increase in blood pressure in diabetic SHR that were untreated or co-treated with t-AUCB (183 ± 5 and 181 ± 2 mmHg respectively). Similarly, non-fasting blood glucose levels were significantly elevated in diabetic SHR compared with control SHR (559 ± 23 compared with 176 ± 6 mg/dl). Inhibition of sEH and/or HO did not significantly lower blood glucose levels in diabetic SHR.

Induction of diabetes significantly increased renal sEH expression compared with control SHR and neither t-AUCB

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**Figure 1**

Cortical sEH expression relative to β-actin (A) and the plasma and cortical EETs/DHETs ratio (B and C) in control and diabetic SHR treated with or without the sEH inhibitor t-AUCB, the HO inhibitor SnMP or both for 4 weeks after 6 weeks of induction of diabetes with streptozotocin.

Inhibition of sEH with t-AUCB increased the plasma and cortical EETs/DHETs ratio without affecting the increase in renal sEH expression in diabetic SHR. Values are means ± S.E.M. (n = 6 per group). *P < 0.05 compared with control SHR, †P < 0.05 compared with diabetic SHR, ‡P < 0.05 compared with diabetic SHR treated with t-AUCB, and #P < 0.05 compared with diabetic SHR treated with SnMP D, diabetic SHR.
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Figure 2 Renal cortical HO-1 and HO-2 expressions relative to \( \beta \)-actin (A and C) and HO-1 activity (B) in control and diabetic SHR with or without t-AUCB, SnMP, or both inhibitors treatment

Inhibition of sEH increased HO-1 expression and activity without affecting HO-2 expression in diabetic SHR whereas co-administration of SnMP with t-AUCB inhibited these changes. Values are means + S.E.M. (n = 6 per group). \( \ast P < 0.05 \) compared with control SHR, \( \neq P < 0.05 \) compared with diabetic SHR and \( \dagger P < 0.05 \) compared with diabetic SHR treated with t-AUCB. D, diabetic SHR.

and/or SnMP treatment affected this increase in diabetic SHR (Figure 1A). We also assessed the levels of EETs and DHETEs in the plasma and renal cortex of diabetic SHR using LC/MS analysis and calculated EETs/DHETEs ratio as indicative of EET availability. We should point out that the 5,6-EET and 5,6-DHETE levels were excluded from our calculation as 5,6-EET regioisomer is very unstable and artifacts are almost a certainty. Induction of diabetes significantly decreased renal cortical EETs levels compared with control SHR (25 + 4 compared with 35 + 4 ng/mg). Inhibition of sEH significantly increased EETs levels in diabetic SHR (42 + 3 ng/mg) and this effect was not affected with co-administration of the HO inhibitor SnMP (40 + 5 ng/mg). We also used the epoxide diol ratio of the EET isomers (8,9-, 11,12- and 14,15-EET) as an indication of EETs bioavailability. In diabetic SHR, the plasma EETs/DHETEs ratio was lower than control SHR although this change was not significant. Inhibition of sEH with t-AUCB significantly increased the plasma EETs/DHETEs ratio in diabetic SHR with or without SnMP treatment when compared with either control or diabetic untreated SHR (\( P < 0.05 \); Figure 1B). In the kidney cortex, the EETs/DHETEs ratio was significantly reduced in diabetic SHR compared with control (\( P < 0.05 \)) and t-AUCB treatment increased the renal cortical EETs/DHETEs ratio in diabetic and diabetic SHR treated with SnMP compared with untreated diabetic SHR (\( P < 0.05 \); Figure 1C).

Because we have shown previously that sEH gene knockout induced HO-1 expression and increased its activity in diabetic mice [9], which could function to protect the kidney from diabetes-induced renal injury via its anti-oxidant and anti-inflammatory properties, we also assessed renal HO-1 expression in control and diabetic SHR treated with t-AUCB, SnMP or both. As shown in (Figure 2A), renal HO-1 expression was significantly elevated in diabetic SHR treated with the sEH inhibitor t-AUCB when compared with the control or diabetic SHR (\( P < 0.05 \)). The increase in renal HO-1 expression was less when the HO inhibitor SnMP was co-administered with t-AUCB compared with t-AUCB-treated diabetic SHR (Figure 2A; \( P < 0.05 \)). Similarly, t-AUCB treatment increased renal cortical HO-1 activity compared with control SHR, and this effect was prevented by co-administration of SnMP (Figure 2B). There was no difference in renal HO-2 expression between groups (Figure 2C).

Urinary albumin and nephrin excretion levels as well as glomerular nephrin expression were used as indicators of renal injury in diabetic SHR. Urinary albumin and nephrin excretion levels were significantly elevated in diabetic SHR compared with the control (\( P < 0.05 \)) and inhibition of sEH with t-AUCB prevented these elevations. Inhibition of HO exacerbated the increase in albuminuria in diabetic SHR and prevented the ability of t-AUCB to lower albumin and nephrin excretion in diabetic SHR (Figures 3A and 3B). However, there was no significant difference in creatinine clearance, an indicator of GFR, between all rat groups (results not shown). The incidence of renal injury and defect in the integrity of the glomerular filtration barrier were further supported by a decrease in glomerular nephrin expression in diabetic SHR compared with control SHR. Inhibition of sEH with t-AUCB restored the decrease in glomerular nephrin expression in diabetic SHR whereas co-administration of the HO inhibitor SnMP prevented the ability of t-AUCB to improve nephrin expression in diabetic SHR (Figure 3B).

The incidence of renal extracellular matrix deposition and fibrosis is now considered a hallmark of diabetic renal injury [7,10]. Accordingly, the progression of renal injury in diabetic
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Figure 3  Urinary albumin (A) and nephrin (B) excretion relative to urinary creatinine \((n = 6–8 \text{ per group})\) and representative images for glomerular nephrin expression (brown staining at \(\times 200, n = 3\)) as indicative of renal injury (B) in control SHR, diabetic SHR, diabetic SHR treated with t-AUCB, SnMP or both t-AUCB and SnMP. Inhibition of sEH significantly reduced the elevation in albumin and nephrin excretion and restored the decrease in glomerular nephrin expression in diabetic SHR and these changes were prevented by co-administration of SnMP. Values are means \(\pm\) S.E.M. \(*P < 0.05\) compared with control SHR, \(\#P < 0.05\) compared with diabetic SHR and \(†P < 0.05\) compared with diabetic SHR treated with t-AUCB. D, diabetic SHR.

SHR was associated with renal cortical fibrosis as manifested by greater interstitial collagen deposition (blue staining, Figure 4A) and greater glomerular and interstitial fibronectin staining (brown staining, Figure 4B) where the staining was more evident in the tubules than in glomeruli of kidney cortex in diabetic SHR. The incidence of renal fibrosis was reduced with sEH inhibition in diabetic SHR whereas co-treatment with the HO inhibitor SnMP prevented the ability of t-AUCB to lower renal fibrosis in diabetic SHR. These findings were supported further by quantitative assessment of glomerular active TGF-\(\beta\) as a marker of renal fibrosis (Figure 4C). Glomerular active TGF-\(\beta\)1 levels were significantly elevated in diabetic compared with control SHR and these changes were reduced with sEH inhibition \((P < 0.05)\). Co-administration of SnMP with t-AUCB prevented the ability of t-AUCB to reduce glomerular TGF-\(\beta\)1 levels in diabetic SHR \((P < 0.05)\).

Because oxidative stress and inflammation contribute to the progression of renal injury in SHR [7,10–12], we also tested whether inhibition of sEH could reduce the elevation in renal inflammation and oxidative stress in diabetic SHR. Renal F4/80, a marker of activated macrophage infiltration, increased in diabetic versus control SHR (Figure 5A). Renal cortical p65-NF-\(\kappa\)B and MCP-1, markers of renal inflammation, were also significantly elevated in diabetic compared with control SHR (Figures 5B and 5C). Inhibition of sEH reduced the elevation in macrophage infiltration and cortical p65-NF-\(\kappa\)B and MCP-1 levels in diabetic SHR \((P < 0.05)\) and these changes were prevented by co-administration of SnMP with t-AUCB. Urinary 8-OHdG, a marker of oxidative stress, was greater in diabetic versus control SHR \((221 \pm 11\) compared with \(94 \pm 12\ ng/day; P < 0.05)\). Inhibition of HO exacerbated the elevation in 8-OHdG excretion \((269 \pm 23\ ng/day; P = 0.08)\) whereas inhibition of sEH did not significantly lower 8-OHdG excretion in diabetic SHR \((209 \pm 21\ ng/day)\). However, t-AUCB prevented SnMP from exacerbating the elevation in 8-OHdG excretion in diabetic SHR treated with both sEH and HO inhibitors \((206 \pm 10\ ng/day)\).

Aside from reducing renal inflammation, we also determined whether the inhibition of sEH reduces systemic inflammation in diabetic SHR by assessing IL-17 expression in the blood from diabetic SHR using flow cytometry. In the diabetic SHR, 9% of blood cells expressed IL-17 compared with only 0.5% of control SHR (Figure 6A). Inhibition of sEH with t-AUCB significantly reduced blood cells expressing IL-17 to 1.5% \((P < 0.05)\). Inhibition of HO with SnMP did not significantly increase the percentage (%) of blood cells expressing IL-17 (3%) nor prevent the t-AUCB-induced decrease in blood cells expressing IL-17 (3.3%) in diabetic SHR (Figure 6A). Immunohistochemical assessment of IL-17 expression in kidney sections also revealed an elevation in IL-17 expression in diabetic SHR and this effect was prevented by co-administration of SnMP (Figure 6B). Using double labelling immunofluorescence (Figure 7), we also showed that IL-17 (green immunofluorescence) was mainly expressed in renal tubular structure. Further, CD3+ immunofluorescence (red), indicative of T-cells, appeared primarily in the renal interstitial space. Nonetheless, foci of yellow staining also appeared with ductal structures of the kidney which may suggest juxtapositioning of tubular cells with CD3+ cells. Consistent with our flow cytometry data, IL-17 expression was markedly elevated in diabetic SHR when compared...
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Figure 4 Representative images (×200) and average score for Masson’s trichrome staining (blue staining, A) and representative images and percentage (%) staining of fibronectin expression (brown staining, B) and glomerular active TGF-β1 levels (C) in control SHR, diabetic SHR, diabetic SHR treated with t-AUCB, SnMP or both
In (A) and (B) n = 3 per group; in (C), n = 6. Inhibition of sEH reduced the elevation in renal fibrosis and glomerular TGF-β1 levels in diabetic SHR and these changes were prevented with co-treatment with SnMP. Values are means ± S.E.M. 
*P < 0.05 compared with control SHR, †P < 0.05 compared with diabetic SHR, and ‡P < 0.05 compared with diabetic SHR treated with t-AUCB.

with control SHR. Importantly, inhibition of sEH decreased IL-17 expression, which was reversed with either SnMP alone or when co-administered with t-AUCB (Figure 7).

DISCUSSION

Our present study determines whether increased EETs availability protects the kidney from renal injury in diabetic SHR in which hypertension coexists with diabetes exacerbating the progression of renal injury and inflammation. Our study also determines whether induction of HO-1 upon inhibition of sEH contributes to the renoprotective effects of EETs in diabetic SHR. Oral administration of the sEH inhibitor, t-AUCB, slowed the progression of renal injury in diabetic SHR. Inhibition of sEH activity increased EETs bioavailability together with induction of HO-1. Further, these changes were associated with reduction in renal fibrosis and decreased NF-κB-induced inflammation. The protective effects of sEH inhibition did not seem to be dependent on blood pressure or blood glucose lowering effects and were not specific for the kidney as inhibition of sEH also decreased IL-17 expression in the blood isolated from diabetic SHR. The protective effect of sEH inhibition is dependent, at least in part, on the induction of HO-1 as inhibition of HO negated the renoprotective effects of sEH inhibition in diabetic SHR. Our data provide evidence for the importance of EETs in slowing the progression of diabetic renal injury and suggest a role of HO-1 in the renoprotective effects of EETs in diabetes.

Increased EETs bioavailability via preventing EETs hydrolysis by sEH using pharmacological inhibition or gene knockout lowers blood pressure and renal injury in an experimental model of hypertension [13–16]. We and others have recently demonstrated that decreased EETs availability in streptozotocin-induced diabetic mice was associated with a marked degree of renal injury and apoptosis and this effect was significantly reduced by increased EETs availability via the inhibition of sEH [9,17]. Inhibition of sEH also reduces hyperglycaemia and pancreatic β-cell islet apoptosis and increases insulin secretion in streptozotocin-induced Type 1 diabetes [18]. Similarly, inhibition of sEH improves insulin signalling in mice and rats fed on a high-fat diet as a model of the metabolic syndrome [19,20]. Sasser et al. [6] previously reported that blood pressure did not change after 10 weeks of streptozotocin-induced diabetes in rats despite a marked degree of renal injury and albuminuria. Thus the present study used the diabetic SHR model which, similar to the clinical manifestation in human, displays coexistence of systemic hypertension and diabetes with a remarkable degree of renal injury. Studies have also shown a marked elevation in renal sEH expression in SHR [21,22] suggesting decreased EETs availability, which makes them an excellent model to determine the effect of sEH inhibition in relation to the progression of diabetic
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Figure 5 Effect of sEH inhibition on renal inflammatory markers in diabetic SHR

(A) Representative images for F4/80 expression as an indicator of activated macrophage infiltration at ×200 and percentage staining of F4/80 respectively (n = 3). (B) and (C) represent renal cortical p65-NF-κB and MCP-1 levels in control and diabetic SHR with or without t-AUCB, SnMP or both treatments (n = 6–8 per group). Inhibition of sEH reduced macrophage infiltration and cortical p65-NF-κB and MCP-1 levels in diabetic SHR and these effects were prevented by co-administration of SnMP with t-AUCB. Values are means ± S.E.M. *P < 0.05 compared with control SHR, †P < 0.05 compared with diabetic SHR, and ‡P < 0.05 compared with diabetic SHR treated with t-AUCB. D, diabetic SHR.

The inhibition of sEH increased the plasma and cortical EETs/DHETEs ratio in diabetic SHR and reduced markers of renal injury but did not significantly attenuate the elevation in blood pressure or blood glucose. Although the same trends are seen in plasma and cortex, it is worth mentioning that the EETs/DHETEs ratio is much higher in kidney cortex than in plasma because DHETEs are predominantly located in plasma due to their higher polarity whereas EETs are largely located in the tissue. Inhibition of sEH has been shown to lower blood pressure in SHR [23,24]; however, t-AUCB did not lower blood pressure in diabetic SHR. Our data are consistent with the previously published data of Olearczyk et al. [25], who showed that sEH inhibition had no effect on mean arterial pressure nor on blood glucose levels in hypertensive Goto–Kakizaki rats, as well as with our recent findings where knocking out of the sEH gene did not lower blood pressure or blood glucose in streptozotocin-induced diabetic mice [9]. Our data suggest that the renoprotective effects of sEH inhibition are independent of blood pressure or blood glucose lowering effects in diabetic SHR. We postulate that failure of sEH inhibitor to lower blood pressure or blood glucose in diabetic SHR could be attributed to the use of t-AUCB as an interventional treatment after signs and symptoms of diabetic complications already exist.

We have demonstrated that induction of HO-1 reduced renal injury and inflammation in diabetic SHR [7] as products of haem metabolism have antioxidant and anti-inflammatory properties; however, the potential toxicity of HO-1 inducers may limit their clinical benefits. EETs and HO share most of their anti-inflammatory properties and links between both have recently been investigated. In vitro, 11,12-EET stimulates HO-1 activity in cultured endothelial cells and 11,12-EET-induced rat mesenteric vasodilation is mediated by increased HO activity [26,27]. In vivo, Sodhi et al. [28] have demonstrated that treatment of HO-2-knockout mice, a model of Type 2 diabetes, with a dual-activity EETs agonist/sEH inhibitor increases renal EETs levels and HO-1 expression together with improvement in insulin sensitivity and reduction in cytokines levels. The same group also showed that EET agonist reduces vascular dysfunction and inflammation via induction of HO-1 in high fat diet fed rats [29]. Deletion of the sEH gene increased HO-1 expression and activity and reduced oxidative stress in streptozotocin-induced diabetic mice [9]. In the present study, inhibition of sEH increased renal HO-1 expression and activity in diabetic SHR and these effects were prevented by co-administration of the HO inhibitor SnMP. SnMP is a competitive inhibitor of microsomal HO, the rate limiting step in the haem catabolic pathway. Several members of the metalloporphyrin family, including SnMP, have been shown to inhibit HO activity, which in turn leads to a decrease in bilirubin concentration. Consistent with our data, previous studies demonstrated that SnMP decreases HO activity in experimental diabetes.
Figure 6  Flow cytometry analysis of IL-17 expression in blood (A), representative images for renal IL-17 expression at ×200 and percentage staining of IL-17 expression (B) in control and diabetic SHR with or without t-AUCB, SnMP or both treatments (n = 3 per group).

Induction of diabetes significantly increased IL-17 expression in SHR blood and this effect was reduced with sEH inhibition. HO inhibition did not significantly affect the expression of IL-17 in blood isolated from diabetic SHR or diabetic SHR treated with t-AUCB. Similarly, inhibition of sEH prevented the increase in renal IL-17 expression in diabetic SHR; this effect was prevented with co-administration of SnMP. Values are means ± S.E.M. *P < 0.05 compared with control SHR, †P < 0.05 compared with diabetic SHR and ‡P < 0.05 compared with diabetic SHR treated with t-AUCB. D, diabetic SHR.

Figure 7  Representative images of double immunofluorescence labelling for CD3 (T-cell marker in red) and IL-17 (green) at ×400 in control and diabetic SHR with or without t-AUCB, SnMP or both treatments (n = 3 per group).

IL-17 was mainly expressed in renal tubules whereas CD3+ immunofluorescence was primarily present in the renal interstitial space. Yellow immunofluorescent staining (grey arrows) also appeared in kidney tubule suggesting juxtapositioning of tubular cells with CD3+ cells. Renal IL-17 expression was markedly elevated in diabetic SHR when compared with control SHR. Importantly, inhibition of sEH decreased IL-17 expression, which was reversed with either SnMP alone or when co-administered with t-AUCB. D, diabetic SHR.
animal models [29–31]. Since hyperglycaemia increases renal oxidative stress and inflammation [7], EETs-mediated HO-1 induction could protect the diabetic kidney via the anti-oxidant and anti-inflammatory effects of the haem metabolites carbon monoxide and biliverdin.

The molecular mechanism(s) by which EETs up-regulate HO-1 is/are yet to be explored. It is well known that HO-1 is transcriptionally activated by hypoxia through HIF-1 (hypoxia-inducible factor-1)-dependent mechanism in the kidney [32–34]. Recent studies also suggest a link between EETs and HIF-1. In astrocytes, hypoxic preconditioning induces CYP2C11 expression, the main arachidonic acid metabolizing epoxygenase to produce EETs, via HIF-1 [35]. Exogenous 11,12-EET increased HIF-1 in human hepatoma cells and human umbilical artery endothelial cells [36]. Inhibition of sEH in H9c2 cells increased HIF-1 DNA binding and delayed the loss of mitochondrial membrane potential caused by ischaemia/reperfusion injury, suggesting its potential cardioprotective effects [37]. Because we have some preliminary data suggesting that the up-regulation of HO-1 upon inhibition of sEH was also associated with elevation in renal HIF-1 expression, we could speculate that EETs induced up-regulation of HO-1 via a HIF-1-dependent mechanism. Future studies will determine the role of HIF-1 in EETs-mediated HO-1 up-regulation.

The earliest clinical evidence of nephropathy is the appearance of low but abnormal levels of albuminuria (≥30 mg/day) referred to as microalbuminuria [1–4]. Diabetic renal injury will then further progress to macro-albuminuria and elevation in blood pressure and once overt nephropathy occurs; the GFR gradually falls over a period of several years leading to end-stage renal disease [1–4]. Inhibition of HO with SnMP was associated with elevation in albuminuria [38,39]. Decreased glomerular nephrin expression and increased nephrinuria are also common markers for increased glomerular injury during diabetes [5,7,40,41]. We previously reported that the increase in albumin and nephrin excretion and the decrease in glomerular nephrin expression were attenuated by induction of HO-1 in diabetic SHR, suggesting a role of HO in protecting kidney from diabetic renal injury [7]. In the present study, t-AUCB reduced the elevation in albumin and nephrin excretion and improved glomerular nephrin expression in SHR without changing creatinine clearance, an indicator of GFR. Co-administration of SnMP with t-AUCB prevented t-AUCB-induced improvement in glomerular nephrin expression and reduction in albumin and nephrin excretion in diabetic SHR. These data are consistent with our recently published data in which sEH inhibition reduced albumin and nephrin excretion in diabetic mice, and inhibition of HO with SnMP reversed these effects [9]. However, in the previous study mice were treated with the sEH inhibitor from the first day of induction of diabetes whereas in the current study rats were treated with the sEH inhibitor and/or HO after diabetic renal injury already exists. These data suggest that inhibition of sEH slows the progression of the early stage signs of diabetic renal injury in SHR, at least in part, through the induction of HO-1, and the renoprotective effect of sEH inhibition in diabetic SHR is not necessarily associated with a change in GFR, a late sign of diabetic renal injury.

Decreased EETs production increases renal collagen deposition and glomerular TGF-β [42], which could exacerbate renal damage in diabetes and hypertension [43,44]. Renal collagen deposition was significantly elevated in streptozotocin-induced diabetic mice and these changes were reduced in diabetic sEH-knockout mice [9]. Inhibition of sEH also lowered glomerular and tubular collagen levels and vascular hypertrophy in salt-sensitive hypertension and diabetic hypertensive rats [16,25]. On the other hand, we recently demonstrated that induction of HO-1 reduced renal TGF-β, collagen and fibronectin expression in diabetic SHR [7]. Consistent with these findings, the current study suggests that induction of HO-1 by sEH inhibition contributes to decreased renal fibrosis in diabetic SHR as inhibition of sEH reduced renal collagen deposition and fibronectin expression together with a reduction in glomerular TGF-β levels and these changes were prevented by co-administration of the HO inhibitor SnMP with t-AUCB.

Oxidative stress and inflammation participate in the progression of diabetic renal injury [7,40,41]. More specifically, NF-κB inflammatory signals are important mediators in the pathogenesis of streptozotocin-induced diabetic injury [45,46]. We have recently shown that increased EETs availability inhibited NF-κB-induced inflammation in streptozotocin-induced diabetic mice [9]. Consistent with our previous findings, inhibition of sEH with t-AUCB reduced the elevation in renal macrophage infiltration, NF-κB activation and MCP-1 levels in diabetic SHR without decreasing oxidative stress, and co-treatment with the HO inhibitor SnMP prevented these changes, suggesting that induction of HO-1 is also involved in EETs anti-inflammatory effects. Our data are also in agreement with previous studies which showed that inhibition of sEH reduced inflammation, macrophage infiltration and NF-κB activation in hypertensive mice and in salt-sensitive hypertensive Goto Kakizaki diabetic rats [25]. The elevation in blood pressure in SHR could drive the elevation in glomerular hydrostatic pressure, which leads to increased albuminuria; however, inhibition of sEH reduced albuminuria without lowering blood pressure in diabetic SHR suggesting that increased EETs bioavailability might drive the decrease in albuminuria via anti-fibrotic and anti-inflammatory properties. The anti-inflammatory effects of EETs is, at least in part, driven by induction of HO, as inhibition of HO with SnMP prevented t-AUCB-induced decreases in renal macrophage infiltration, NF-κB activation and MCP-1 levels in diabetic SHR. The ability of sEH inhibitor to reduce inflammation is not kidney specific as t-AUCB treatment reduced the increase in IL-17 expression in blood as well as in the kidney from diabetic SHR where elevated IL-17 expression is used as a marker of inflammation in diabetic patients [47]. Surprisingly, inhibition of HO with SnMP alone or even when used in combination with t-AUCB did not exacerbate IL-17 expression in blood isolated from diabetic SHR; however, it prevented the t-AUCB ability to lower renal IL-17 expression in diabetic SHR. These data suggest that HO inhibition could prevent renal but not systemic EETs anti-inflammatory properties. The ability of SnMP to inhibit HO activity and increase immune cell infiltration without a significant change in NF-κB activation could be attributed to its inability to further exacerbate hypertension in diabetic SHR. However, our data clearly showed that co-administration of SnMP with t-AUCB inhibited the ability of t-AUCB to significantly reduce NF-κB inflammatory signalling activation.
CLINICAL PERSPECTIVES

- Our data suggest that increased EETs levels, via interventional treatment with the sEH inhibitor t-AUCB, slows the progression of renal injury in diabetic SHR. The renoprotective effects of sEH inhibition in diabetes appear to be independent of blood pressure or blood glucose lowering effects and might be attributed to a reduction in NF-κB-induced inflammation and decreased renal fibrosis.

- The mechanism by which sEH inhibition reduces renal injury could be directly through increased EETs anti-inflammatory effects or indirectly via induction of HO-1 activity which also has anti-inflammatory properties. It is also clear that antagonism of HO reduces the renoprotective effects of sEH in diabetic SHR. This could be attributed to the reduction in the anti-inflammatory effects of EETs upon inhibition of HO.

- These findings support the hypothesis that reducing inflammation via increased EETs availability could be an alternative approach to traditional anti-inflammatory therapies for treatment of diabetic renal injury. Moreover, inducers of HO-1 could be synergized with inhibitors of sEH to prevent diabetic renal deterioration.

AUTHOR CONTRIBUTION
Ahmed Elmarakby conceived and designed the experiments, analysed the data and wrote the paper; Jessica Faulkner, Chelsey Pye, Katelyn Rouch and Adulmohsin Alhashim performed the experiments and proofed the paper; Babak Baban performed and analysed the flow cytometry and helped in drafting the paper; Krishna Rao Maddipati determined the plasma and renal levels of EETs and DHETEs using LC/MS and helped in drafting the paper.

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