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Administration of a substituted adamantyl urea inhibitor of soluble epoxide hydrolase protects the kidney from damage in hypertensive Goto–Kakizaki rats

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ABSTRACT

Hypertension and Type 2 diabetes are co-morbid diseases that lead to the development of nephropathy. sEH (soluble epoxide hydrolase) inhibitors are reported to provide protection from renal injury. We hypothesized that the sEH inhibitor AUDA [12-(3-adamantan-1-yl-ureido)-dodecanoic acid] protects the kidney from the development of nephropathy associated with hypertension and Type 2 diabetes. Hypertension was induced in spontaneously diabetic GK (Goto–Kakizaki) rats using AngII (angiotensin II) and a high-salt diet. Hypertensive GK rats were treated for 2 weeks with either AUDA or its vehicle added to drinking water. MAP (mean arterial pressure) increased from 118 ± 2 mmHg to 182 ± 20 and 187 ± 6 mmHg for vehicle and AUDA-treated hypertensive GK rats respectively. AUDA treatment did not alter blood glucose. Hypertension in GK rats resulted in a 17-fold increase in urinary albumin excretion, which was decreased with AUDA treatment. Renal histological evaluation determined that AUDA treatment decreased glomerular and tubular damage. In addition, AUDA treatment attenuated macrophage infiltration and inhibited urinary excretion of MCP-1 (monocyte chemoattractant protein-1) and kidney cortex MCP-1 gene expression. Taken together, these results provide evidence that sEH inhibition with AUDA attenuates the progression of renal damage associated with hypertension and Type 2 diabetes.

INTRODUCTION

Hypertension is a major risk factor for the development of nephropathy in patients with Type 2 diabetes [1]. If left untreated, hypertension exacerbates the progression of nephropathy to ESRD (end-stage renal disease), which is the leading cause of mortality in patients with Type 2 diabetes. It has been estimated that more than 3 million people in the United States have hypertension and diabetes and that these disease states are co-morbid [1].

Key words: blood pressure, diabetes, eicosanoid, inflammation, nephropathy, soluble epoxide hydrolase.

Abbreviations: AngII, angiotensin II; AUBA, 4-(3-adamantan-1-yl-ureido)-butanoic acid; AUDA 12-(3-adamantan-1-yl-ureido)-dodecanoic acid; AUDA-nBE, AUDA *n*-butyl ester; BP, blood pressure; CDU, 1-cyclohexyl-3-dodecylurea; DOCA, deoxycorticosterone acetate; EET, epoxyeicosatrienoic acid; EH, epoxide hydrolase; ESRD, end-stage renal disease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GK rat, Goto–Kakizaki rat; HR, heart rate; MAP, mean arterial pressure; MCP, monocyte chemoattractant protein; NF-κB, nuclear factor κB; PPAR-α, peroxisome-proliferator-activated receptor-α; sEH, soluble EH; TNF-α, tumour necrosis factor-α.

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Although the pathological progression of nephropathy is defined clinically, the initiating and propagating factors that lead to the development of nephropathy and its progression to ESRD have yet to be elucidated. A number of metabolic and haemodynamic factors have been identified that could contribute to the decline in renal function. Previous findings have also implicated the involvement of a subacute inflammatory component that is characterized by an increase in the infiltration of pro-inflammatory cells into renal tissue and an increase in the transcription of NF-κB (nuclear factor κB)-mediated pro-inflammatory genes [2,3]. This inflammatory response can result in structural alterations within the kidney that perturb glomerular function and lead to an increase in glomerular filtration rate, all of which occur early in the progression of nephropathy to ESRD.

Epoxygenase metabolites and their regulation by the sEH [soluble EH (epoxide hydrolase)] enzyme have been reported to regulate renal blood flow and BP (blood pressure) [4–6]. In addition to maintaining renal homoeostasis, the epoxides, EETs (epoxyeicosatrienoic acids) and EH inhibition are reported to prevent inflammation by inhibiting the expression of cell adhesion molecules and suppressing leucocyte adhesion [7,8]. Interestingly, increasing epoxygenase metabolite levels or sEH inhibition has been demonstrated to provide cardiovascular and renal protection. Given the renal-protective and anti-inflammatory properties, inhibition of sEH has been identified as a target for the treatment of diseases such as hypertension and inflammation [9]. Pharmacological inhibitors of sEH bind competitively and with high affinity to the catalytic site of sEH, inhibiting its enzymatic activity [10]. These urea-based sEH inhibitors have been modified to allow for oral administration [11]. Thus we hypothesized that sEH inhibition could protect the kidney from the development of nephropathy associated with hypertension and Type 2 diabetes.

In the present studies, we administered the sEH inhibitor AUDA [12-(3-adamantan-1-yl-ureido)-dodecanoic acid] for 2 weeks in hypertensive GK (Goto–Kakizaki) Type 2 diabetic rats. We propose that the protection afforded the kidney results, at least in part, by inhibiting the inflammatory component of nephropathy. We examined the effects of AUDA, an orally active EH inhibitor on MAP (mean arterial pressure) and HR (heart rate). In separate experiments, we investigated the effects of AUDA on renal injury and the infiltration of pro-inflammatory cells into the kidney.

MATERIALS AND METHODS

Animals

The Medical College of Georgia Animal Care and Use Committee approved all of the animal protocols. At 12 weeks of age, male GK rats (Taconic) were divided into three experimental groups: (i) GK control, which served as a control and received a normal salt diet and tap water; (ii) GK rats made hypertensive and receiving vehicle treatment; and (iii) GK rats made hypertensive and receiving AUDA dissolved in the drinking water. Age-matched male Wistar rats (Taconic) were included as a comparison group. Hypertension was induced by continuous infusion of AngII (angiotensin II; 65 ng/min) via an osmotic mini-pump (model 2004; Alzet). Rats from this group were also fed a high-salt diet containing 8% (w/v) NaCl. AUDA (25 mg/l) was dissolved in a drinking water solution, as described previously [12]. Rats from all of the groups were allowed access to food and water *ad libitum* during the 14-day treatment period.

Measurement of BP

Telemetry transmitters (Data Sciences) were implanted and data collected, as described previously [13]. MAP and HR were measured once every 5 min for the duration of the experimental protocol. Day and night time averages were calculated and plotted. The Biotelemetry Core at the Medical College of Georgia provided assistance with telemetry studies.

Measurement of urinary electrolytes, albumin and MCP (monocyte chemoattractant protein)-1

Rats were housed in metabolic cages, which separate urine from food and faeces, 24 h prior to the completion of the treatment period. Urine was collected in a tube containing 5 mg of triphenylphosphine. Urine volumes were measured, and the urine aliquoted and stored at −80 °C until analysed. Concentrations of urinary electrolytes (Na^+, Cl^- and K^+) were measured using ion-selective electrodes (Synchron EL-ISE; Beckman Instruments). Albumin (Exocell) and MCP-1 (BD Biosciences) concentrations were measured using ELISAs.

Measurement of plasma insulin, cholesterol and triacylglycerols (triglycerides)

Whole blood was collected into a heparanized syringe and transferred to a centrifuge tube. The uncoagulated blood was spun for 5 min at 1000 *g* to sediment red blood cells. The plasma was pipetted off and aliquoted. Aliquots were stored at −80 °C until assayed. Plasma insulin was measured using an ELISA (Alpco Diagnostics). Cholesterol and total triacylglycerols were measured using a colorimetric assay (WAKO Chemicals).

Histology and immunohistochemistry

At the end of the treatment period, kidneys were isolated and perfused with ice-cold physiological salt solution [125 mmol/l NaCl, 5.0 mmol/l KCl, 1.0 mmol/l $MgCl_2$, 10.0 mmol/l glucose, 20.0 mmol/l Hepes, 1.8 mmol/l $CaCl_2$ and 0.111 g/l BSA (pH 7.4)], followed by a 10%

(w/v) formalin solution to fix the kidney tissue. After perfusion, the kidneys were removed, cut and fixed in 10% (w/v) formalin solution overnight. The kidney sections were embedded in paraffin and cut into 4-μm slices for use in the histology and immunohistochemistry protocols. For histology, formalin-fixed paraffin-embedded kidney slices were deparaffinized, re-hydrated and stained with haematoxylin/eosin. Another series were stained using a Masson's Trichrome kit according to the manufacturer's protocol (Richard Allan Scientific). For immunohistochemistry, deparaffinized re-hydrated kidney slices were incubated with a 10% (v/v) H_2O_2 solution in methanol to inhibit endogenase peroxidase activity and were then blocked with normal goat serum. Kidney slices were then incubated with a primary antibody that recognizes monocytes/macrophages (mouse anti-rat CD68). Sections were incubated with anti-(mouse IgG) secondary antibody conjugated with HRP (horseradish peroxidase) and visualized using the chromogen diaminobenzamine. Slides were counterstained with haematoxylin.

Evaluations of renal damage from haematoxylin/eosin-stained sections were performed without the evaluator having knowledge of the treatment groups. Kidney sections were scored using the following numeric scale: 0 = no damage; +1 = very mild; +2 = mild; +3 = moderate; and +4 = severe. Evaluation of fibrosis and renal injury were evaluated further in Masson's-Trichrome-stained kidney sections. Stained sections were visualized by light microscopy and representative digital images of five cortex and three medulla areas were obtained for each kidney. To quantify the Masson's Trichrome staining, the eight random images from each kidney were assigned random numbers and scored by three blinded observers on a scale of 0–10 for collagen deposition, fibrosis and renal injury. Semi-quantitative evaluation of the renal inflammatory cell infiltration was also performed without the evaluator having knowledge of the treatment groups. The numbers of CD68-positive cells were counted from a given area of kidney, and the numbers obtained from each treatment group averaged and plotted.

Measurement of AUBA [4-(3-adamantan-1-yl-ureido)-butanoic acid] levels

To confirm AUDA in the treated groups, urinary levels of the inactive AUDA metabolite AUBA was measured in the urine, as described previously [14,15]. Briefly, the analytes were separated from other components in the urine by solid-phase extraction using a conditioned Oasis® HLB SPE cartridge. The analytes were eluted from the column with ethyl acetate. Ethyl acetate was evaporated under nitrogen and the resulting residue was suspended in 50 μl of methanol-containing internal standards. The samples were separated using HPLC. Analyte concentrations were determined by comparison with a measured standard curve.

Measurement of blood glucose

Non-fasting blood glucose was measured from blood obtained by tail prick. The amount of glucose in the blood was determined using the Accu-chek Advantage blood glucose monitoring system (Roche).

Real-time PCR

Total RNA was prepared from kidney cortex using ultrapure TRIzol® reagent, according to the manufacturer's instructions (Gibco-BRL). Reverse transcription was performed on equal amounts of total RNA (3 μg) using a blend of oligo(dT) primers and random hexanucleotide primers to produce a cDNA library for each sample. Real-time PCR reactions were run on an iCycler iQ Real-Time PCR Detection System using iQ Supermix (Bio-Rad Laboratories), which is optimized for real-time PCR applications. TaqMan probes (Roche Molecular Systems) and oligonucleotide primers were designed from the published cDNA sequences of MCP-1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) using Beacon Designer software (Premier Biosoft International). Each sample was run in duplicate and the comparative C_t method was used to quantify the fold increase compared with controls. Probes and primer sequences were used as follows: MCP-1 probe, 5′-FAM-CAC CTG CTG CTA CTC ATT CAC TGG C-BHQ-3′ [where FAM and BHQ are 6-carboxyfluorescein and Black Hole Quencher respectively]; MCP-1 forward, 5′-CAG CCA GAT GCA GTT AAT GC-3′; MCP-1 reverse, 5′-GCT TGG TGA CAA ATA CTA CAG C-3′; GAPDH probe, 5′-FAM-ACT CCA CGA CAT ACT CAG CAC CAG CA-BHQ-3′; GAPDH forward, 5′-CAC GGC AAG TTC AAC GGC-3′; and GAPDH reverse, 5′-GGT GGT GAA GAC GCC AGT A-3′.

NF-κB transcription factor assay

Whole-cell lysates were obtained from the kidney cortex using a nuclear extract kit (Active Motif). Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce). A total of 20 μg of whole-cell extract was used for the determination of NF-κB activity using the TransAM NFκB p65 Transcription Factor assay kit (Active Motif). Each of the standards and samples were run in duplicate. In addition to the Jurkat cell nuclear extract provided in the kit as a positive control, HeLa cell whole-cell lysates from cells cultured for 5 min in the presence and absence of 10 μg/ml TNF-α (tumour necrosis factor-α) and 10 μmol/l calyculin A were also run. To ensure NF-κB specificity, HeLa cell whole-cell lysate from cells treated with TNF-α and the whole-cell lysate obtained from experimental groups were run in the presence of the wild-type NF-κB consensus oligonucleotide and mutated NF-κB consensus oligonucleotide.

The wild-type consensus oligonucleotide completely blocked NF-κB binding with absorbance that was not different from the blank wells (no NF-κB p65 standard). Conversely, the mutated consensus oligonucleotide was without effect on NF-κB binding (results not shown). The amount of activated NF-κB was normalized per μg of cortical protein used in the assay.

Statistics

All results are presented as means $\pm$ S.E.M. Statistical significance between experimental groups was determined using ANOVA. In the event that the associated F ratio indicated that changes occurred, a least-significant-difference test was used to identify individual differences. P values of 0.05 or less were considered statistically significant.

RESULTS

Urinary excretion of AUDA and AUBA were measured to confirm proper sEH inhibition. AUDA excretion averaged 28 $\pm$ 9 ng/day, and AUBA excretion averaged 24049 $\pm$ 6234 mg/day in hypertensive GK rats after 14 days of AUDA administration.

It is well documented that a major risk factor in the development and progression of nephropathy in patients with Type 2 diabetes is hypertension [16,17]. Our laboratory has reported that sEH inhibition lowered BP and provided renal protection in an animal model of hypertension [13,18]. To determine whether sEH inhibition lowers BP and/or protects the kidney from the renal damage associated with hypertension in Type 2 diabetes, AUDA was administered to hypertensive GK rats. Arterial BP and HR are shown in Figures 1(A) and 1(B) respectively. Treatment of hypertensive GK rats with AUDA did not lower arterial BP (182 $\pm$ 20 compared with 187 $\pm$ 6 mmHg for vehicle- and AUDA-treated hypertensive GK rats respectively). The arterial BP of GK control rats was 118 $\pm$ 2 mmHg. AUDA treatment did not alter HR in hypertensive GK rats. As predicted, hypertension resulted in an initial decrease in the HR of GK rats, with the HR reaching its lowest point after 5 days (280 $\pm$ 2 beats/min for the vehicle-treated hypertensive GK group and 307 $\pm$ 3 beats/min for the AUDA-treated hypertensive GK group compared with 368 $\pm$ 12 beats/min in GK controls on day 5). After day 5, the HR of the vehicle- and AUDA-treated hypertensive GK rats increased and was, on average, significantly higher than the GK control rats (446 $\pm$ 4 and 448 $\pm$ 3 beats/min for the vehicle- and AUDA-treated hypertensive GK groups respectively, compared with 357 $\pm$ 4 beats/min for the GK control group).

In separate studies, body weight, urine volumes and urine electrolytes were measured at the end of the 2-week treatment period, as well as blood glucose, plasma insulin,

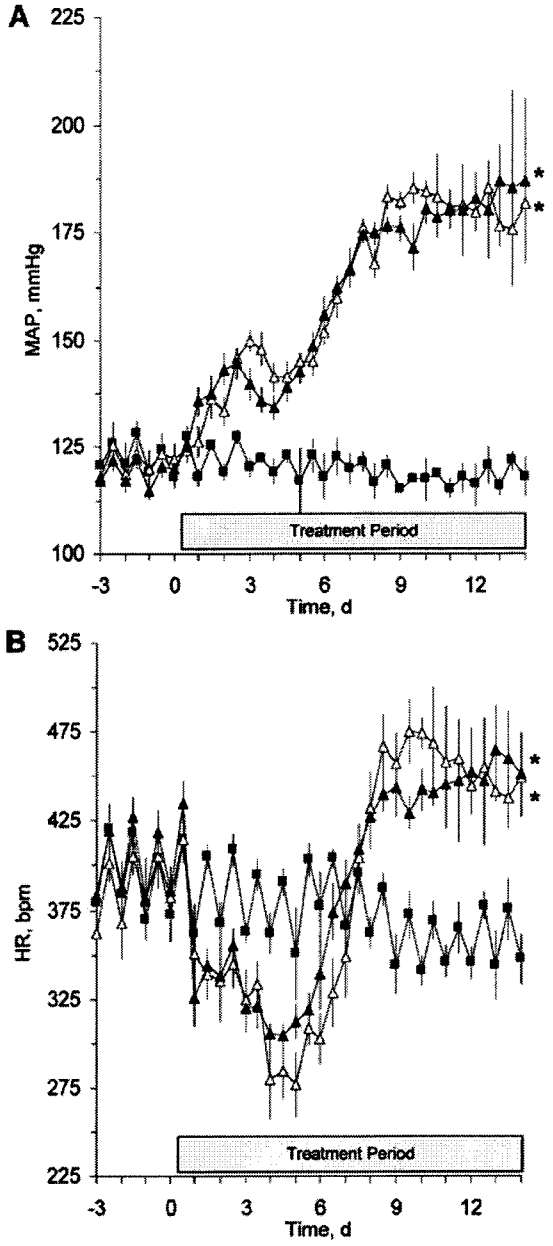

Figure 1 Effect of AUDA on MAP (A) and HR (B) in hypertensive GK rats ($n = 4$)

Data points represent 12-h day and night averages of MAP and HR in GK control rats (■), vehicle-treated hypertensive GK rats (△) and AUDA-treated hypertensive GK rats (▲). Values represent means $\pm$ S.E.M. *$P < 0.05$ compared with GK control rats.

total cholesterol and triacylglycerols. These results are summarized in Table 1. As expected, the GK control group had elevated non-fasting blood glucose levels, which is consistent with the GK rat as a model of Type 2 diabetes. The GK control group also had significantly decreased plasma insulin, and these results are congruous with impaired insulin secretion and impaired development of pancreatic islet cells, both of which are

Table 1 Metabolic parameters in the three groups of GK rats used in the present study ($n = 9$–12 rats/group)

Values are means ± S.E.M. *$P < 0.05$ compared with the GK control group. GK rats were made hypertensive by treating with AngII and a high-salt diet, as described in the Materials and methods section.

Parameter	GK control	Hypertensive GK rats treated with	
		Vehicle	AUDA
Body weight (g)	357 ± 10	292 ± 14*	280 ± 9*
Urine volume (ml)	16 ± 1	88 ± 7*	79 ± 12*
Urine electrolytes (mmol/day)			
Na^+	0.9 ± 0.1	17.0 ± 1.4*	13.7 ± 2.3*
K^+	3.5 ± 0.2	2.3 ± 0.2*	1.9 ± 0.3*
Cl^-	1.6 ± 0.1	17.5 ± 1.4*	13.9 ± 2.3*
Non-fasting blood glucose (mg/dl)	176 ± 24	138 ± 14	144 ± 15
Plasma insulin (μg/l)	0.8 ± 0.1	0.2 ± 0.1*	0.2 ± 0.1*
Cholesterol (mg/dl)	67.7 ± 5.3	74.0 ± 9.6	76.1 ± 9.7
Triacylglycerols (mg/dl)	30.2 ± 9.7	24.1 ± 7.2	23.7 ± 6.8

involved in the pathogenesis of diabetes in the GK rat [19–21]. Although the GK control and hypertensive groups had elevated blood glucose, plasma cholesterol and triacylglycerol levels, AUDA treatment did not change these levels in hypertensive GK rats. Hypertensive GK rats had a significant decrease in body weight and a significant increase in urine volume compared with the GK control group. The increased urinary volume was accompanied by an expected increase in Na^+ and Cl^- excretion resulting from the AngII- and high-salt diet-induced hypertension. Urinary electrolyte excretion was not altered in the hypertensive GK rats administered AUDA.

To investigate whether or not sEH inhibition could prevent renal damage associated with hypertension and Type 2 diabetes, urinary excretion of albumin was measured, an indicator of renal damage. Urinary albumin excretion averaged 0.9 ± 0.2 mg/day at day 0, 2.5 ± 0.4 mg/day at day 7 and 1.4 ± 0.2 mg/day at day 14 in GK controls and, at the end of the experimental period, this level was comparable with that observed in Wistar rats (1.0 ± 0.4 mg/day). Induction of hypertension in GK rats resulted in an increase in urinary albumin excretion at day 7 (8.6 ± 2.6 mg/day) and a 17-fold increase to 22.0 ± 5.0 mg/day at day 14, indicating the development of renal damage (Figure 2). AUDA treatment inhibited hypertension-induced increases in urinary albumin excretion at day 7 (2.6 ± 1.6 mg/day) and day 14 (6.3 ± 2.6 mg/day).

To determine the extent of the renal damage (Figure 3(A), semi-quantitative analyses of histological sections were made. Representative sections are provided in Figures 2(B) and 3(B). The GK control group had very mild tubular dilation (+1), tubular atrophy (+1) and

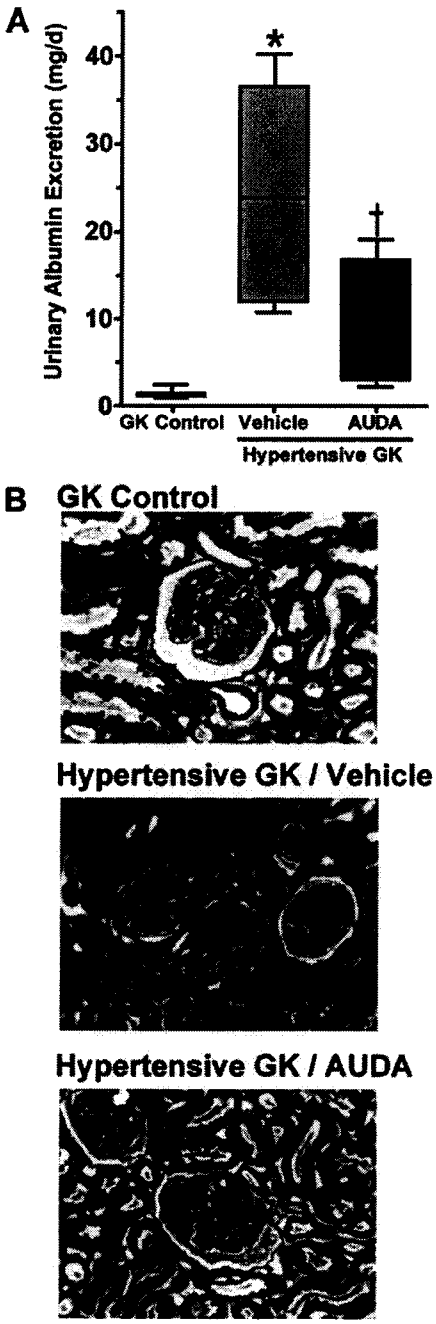

Figure 2 Effects of AUDA treatment on (A) urinary albumin excretion and (B) kidney morphology from hypertensive GK rats ($n = 6$–9)

*$P < 0.05$ compared with GK control and AUDA-treated hypertensive GK groups; †$P < 0.05$ compared with the GK control group. In (B), sections were stained with haemotoxylin/eosin. Magnification, × 400.

glomerular hypertrophy (+1). This is comparable with Wistar rats that did not have any damage, sclerosis or necrosis of glomeruli detected in histological sections (results not shown). Vehicle-treated hypertensive GK rats had mild-to-moderate renal injury with increased fibroid

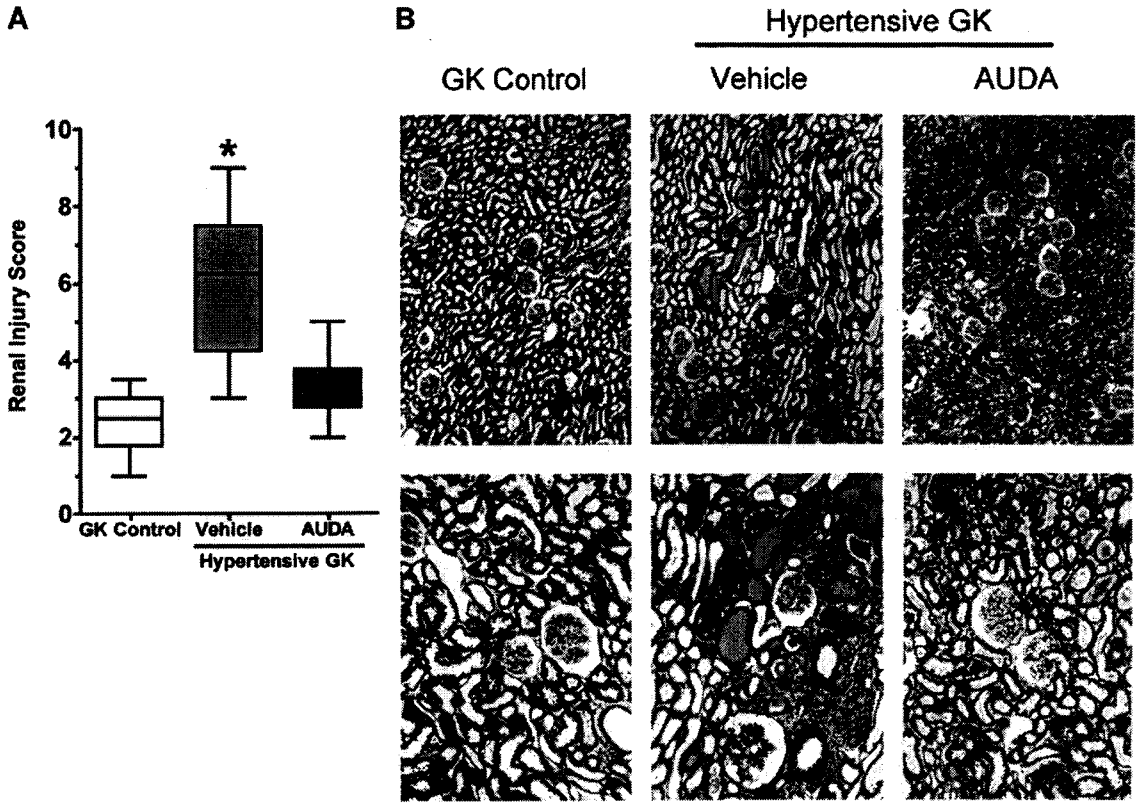

Figure 3 Effects of AUDA treatment on (A) renal fibrosis and injury score and (B) kidney morphology in hypertensive GK rats ($n = 4$)

*$P < 0.05$ compared with GK control and AUDA-treated hypertensive GK groups. In (B), sections were stained with Masson's Trichrome. Upper panels, magnification, × 50; lower panels, magnification, × 400.

necrosis (+3) and hyaline arteriopathy of the interstitial arterioles (+3), increased tubular dilation (+3), tubular atrophy (+2) and cast formation (+2), and increased mononuclear cell infiltration (+2). Hypertension did not alter the glomerular hypertrophy present in the GK control group. Treatment with AUDA reduced hypertension-induced renal injury scores for fibroid necrosis (+1) and hyaline arteriopathy of the interstitial arterioles (+1). AUDA treatment also decreased tubular dilation (+2), atrophy (+1) and cast formation (+1), as well as decreasing the score for mononuclear cell infiltration (+1); however, glomerular hypertrophy scores were unchanged. Masson's Trichrome staining was evaluated to assess the extent of renal fibrosis and injury. As shown in Figure 3(B), there was extensive fibrosis and renal injury in the vehicle-treated hypertensive GK group which was ameliorated by AUDA treatment. Taken together, these results provide support for the hypothesis that inhibition of the sEH protects the kidney from renal damage associated with hypertension and Type 2 diabetes, independent of any affects on BP.

The progression of nephropathy associated with hypertension and Type 2 diabetes involves an inflammatory component that is characterized by increased expression of pro-inflammatory genes and infiltration of pro-inflammatory cells into the kidney interstitium. sEH inhibition has been identified as a target for the treatment of inflammatory diseases [22,23]. To determine whether sEH inhibition decreases the infiltration of pro-inflammatory cells into the kidney, the infiltration of monocyte/macrophages was measured. The GK control group averaged 1.3 ± 0.3 monocytes/mm^2 and Wistar rats averaged 1.6 ± 0.4 monocytes/mm^2. The number of monocytes/macrophages in the medulla were also determined and found not to be different between any of the treatment groups (results not shown). Inducing hypertension in GK rats resulted in a 4-fold increase in monocyte/macrophage infiltration into the kidney cortex (Figure 4B). Interestingly, this increase in monocyte/macrophage infiltration corresponded with the measured increase in urinary albumin excretion (Figure 2). Treatment of hypertensive GK rats with AUDA significantly attenuated the hypertension-induced increase in monocyte/macrophage infiltration into the kidney cortex.

Activation and infiltration of monocytes/macrophages from the bloodstream into the tissue results in part from

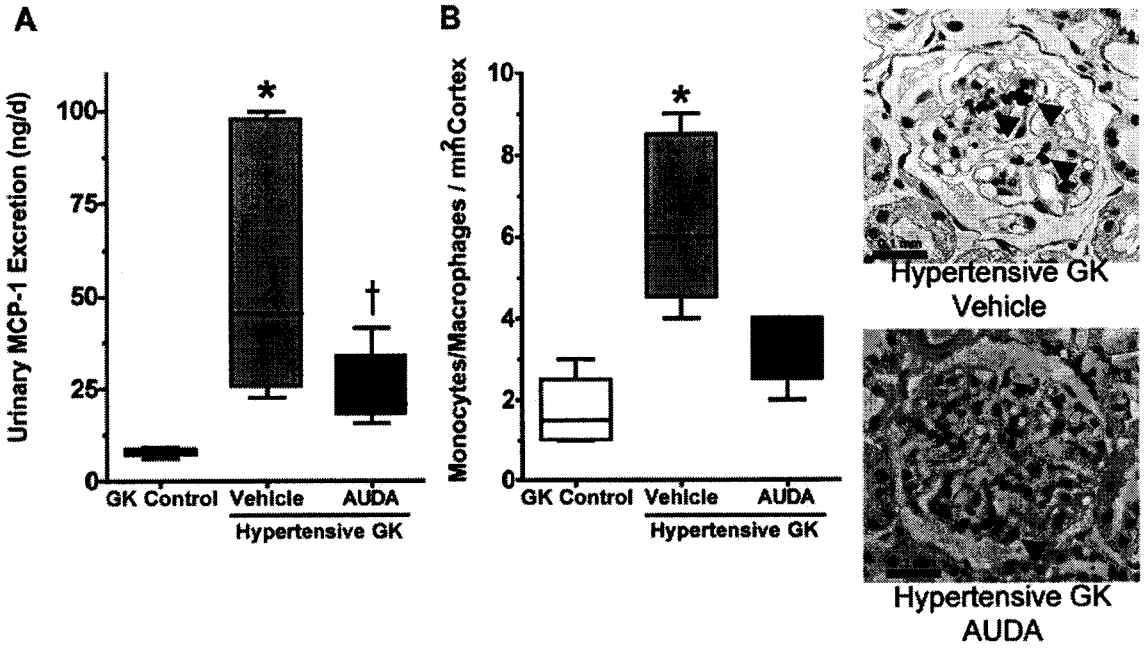

Figure 4 Effects of AUDA treatment on (A) urinary excretion of MCP-1 and (B) infiltration of pro-inflammatory cells into the kidney cortex of hypertensive GK rats ($n = 6$–7)

*$P < 0.05$ compared with GK control and AUDA-treated hypertensive GK groups. †$P < 0.05$ compared with the GK control group. In the right-hand panels of (B), sections were incubated with CD68 antibodies, followed by secondary antibody and visualized using the chromogen diaminobenzamine. Sections were counterstained with haematoxylin. Arrows indicate the infiltrating pro-inflammatory cells. Magnification, × 400.

increased transcription and production of MCP-1. To determine whether the increased infiltration of monocyte/macrophages into the kidney resulted from an increase in MCP-1 protein, urinary MCP-1 excretion was measured. As shown in Figure 4(A), the GK control group had low levels of MCP-1 in the urine. Urinary excretion of MCP-1 was significantly increased in the vehicle-treated hypertensive GK group. AUDA treatment attenuated hypertension-induced increases in urinary MCP-1 excretion. These findings are in agreement with the infiltration of monocytes/macrophages into the kidney. To determine whether the increase in urinary MCP-1 excretion resulted from an increase in MCP-1 transcription, kidney cortex was obtained and the amount of MCP-1 mRNA measured by PCR. Cortex from the GK control group had low levels of MCP-1 gene expression ($2^{-\Delta\Delta Ct} = 1.21 \pm 0.29$). The vehicle-treated hypertensive GK group had a 3.4-fold increase in MCP-1 gene expression in the kidney cortex ($2^{-\Delta\Delta Ct} = 3.57 \pm 0.43$), which was inhibited to control values with AUDA treatment ($2^{-\Delta\Delta Ct} = 1.14 \pm 0.27$). Consistent with the other inflammatory results, renal cortical NF-κB activity was significantly higher in the vehicle-treated hypertensive GK group and AUDA treatment decreased NF-κB activity in these rats (Figure 5). Taken together, these results suggest that inhibition of the sEH inhibits renal damage by inhibiting the infiltration of pro-inflammatory cells into the kidney associated with hypertension and Type 2 diabetes.

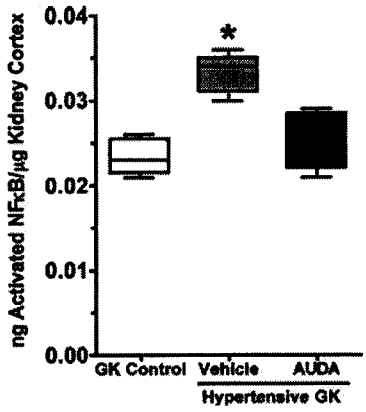

Figure 5 Effects of AUDA treatment on renal NF-κB activity in GK rats ($n = 6$–8)

*$P < 0.05$ compared with GK control and AUDA-treated hypertensive GK groups.

DISCUSSION

Hypertension is a major risk factor for patients with Type 2 diabetes. It has been proposed that hypertension and Type 2 diabetes are co-morbid disease states and that the development of hypertension exacerbates the progression of diabetic nephropathy to ESRD. An inflammatory response has been identified that contributes

to this progression. This subacute inflammatory response is characterized by an increase in the infiltration of pro-inflammatory cells into the kidney and an increase in the expression of pro-inflammatory genes [2,3]. sEH has been identified as a target for the treatment of hypertension and inflammation. Inhibitors of sEH have been reported to decrease BP in hypertension and decrease hypertension-induced renal damage [13,24]. In addition, sEH inhibitors have anti-inflammatory properties and were reported to decrease inflammation-induced tissue damage [22,23]. In the present study, we investigated the hypothesis that sEH inhibition protects the kidney from renal damage associated with hypertension and Type 2 diabetes. Hypertension induced in the spontaneously Type 2 diabetic GK rat accelerated the development of renal damage and the infiltration of pro-inflammatory cells into the kidney. AUDA treatment decreased renal damage, independent of an effect on BP. In addition, AUDA treatment inhibited the infiltration of pro-inflammatory cells into the kidney and decreased the expression of pro-inflammatory genes.

In the present studies, MAP increased significantly in GK rats infused with AngII and fed a high-salt diet. The oral administration of AUDA to hypertensive GK rats had no effect on MAP. This result was of particular interest because the administration of AUDA or other sEH inhibitors were reported to decrease BP in animal models of hypertension [13,18]. Our laboratory has demonstrated that AUDA treatment decreased BP in hypertensive Sprague–Dawley rats. In these experiments hypertension was induced with either AngII infusion or AngII infusion with a high-salt diet [25]. The urinary excretion of AUDA averaged 121 ± 61 ng/day for the AUDA-treated AngII/high-salt-diet-induced hypertensive group. In the present study, the urinary excretion of AUDA averaged only 28 ± 9 ng/day for the AUDA-treated hypertensive GK group. Therefore it is possible that higher AUDA levels than those attained in the present study are required to elicit effects on BP. Although AUDA did not alter MAP, AUDA treatment did have beneficial effects on the hypertensive GK rats. Therefore the results in the present study suggest that the renal protective effects afforded by AUDA occur via a mechanism that is independent of its effects on BP.

Hypertension induced in the GK rat resulted in an increase in BP and was accompanied by a measured increase in HR during the second week. It has been reported that AngII and/or salt-sensitive hypertension results in a transient decrease in HR that returns to control levels by the end of the first week [25]. Therefore the ability to control HR in the spontaneously Type 2 diabetic GK rat after the induction of hypertension using AngII and a high-salt diet is not the same as rats with hypertension alone. The specific contribution of increased HR to the development of renal damage in this animal model of hypertension and Type 2 diabetes is not known.

The progression of renal damage in spontaneously diabetic GK rats was accelerated by hypertension. This was shown by an increase in urinary albumin excretion, an indicator of renal damage. In addition, hypertension induced morphological changes in the kidney that are characteristic of the development of nephropathy, such as tubular dilation and fibroid necrosis of interstitial arterioles. Similar changes were observed in other models of hypertension induced in the GK rat. Salt-sensitive hypertension resulted in a 50 % increase in albuminuria and administration of DOCA (deoxycorticosterone acetate) salt to GK rats, a model of mineralcorticoid-induced experimental hypertension, induced a 4-fold increase in proteinuria [26,27]. Likewise, hemi-nephrectomized GK rats have increased macrophage infiltration and accelerated renal damage [28]. Treatment of hypertensive GK rats with AUDA inhibited hypertension-induced increases in albumin excretion and prevented the development of many of the morphological changes induced by hypertension. These renal protective effects resulting from EH inhibition are consistent with findings generated in our laboratory using *in vivo* models of hypertension. Zhao et al. [13] reported that chronic administration of the EH inhibitor CDU (1-cyclohexyl-3-dodecylurea) to AngII-induced hypertensive Sprague–Dawley rats decreased urinary albumin excretion and protected the kidney from hypertension-induced damage. Similarly, it was reported that AUDA decreased urinary albumin excretion and renal damage in both AngII-induced and AngII-induced salt-sensitive hypertensive Sprague–Dawley rats [25]. Taken together, these results provide support for the hypothesis that AUDA protects the kidney from damage associated with hypertension and Type 2 diabetes.

An inflammatory component has been identified that contributes to the progression of nephropathy to ESRD. This subacute inflammatory component is characterized by an increase in the infiltration of inflammatory cells into the kidney and an increase in the expression of pro-inflammatory genes. In the present study, we demonstrated that hypertension exacerbated monocyte/macrophage infiltration in the GK rat kidney. This increased infiltration of inflammatory cells into the kidney cortex was accompanied by an increase in urinary MCP-1 excretion and an increase in MCP-1 gene expression in the kidney cortex. This hypertension-induced increase in the subacute inflammatory response corresponds with the observed increase in urinary albumin excretion and renal morphological damage. These findings are consistent with renal damage that occurs in other experimental models of hypertension and hemi-nephrectomy in the GK rat [26–28]. Cheng et al. [26] reported an increase in monocyte/macrophage infiltration into the kidney with a corresponding increase in immunostaining for ICAM-1 (intracellular adhesion molecule-1) in GK rats with salt-sensitive hypertension. In addition, administration of

DOCA salt to GK rats resulted in an increase in macrophage infiltration into the kidney and was accompanied by an increase in renal immunohistochemical staining for MCP-1 [28]. Interestingly, treatment of hypertensive GK rats with AUDA inhibited the infiltration of monocyte/macrophages into the kidney and decreased gene expression and urinary excretion of MCP-1. Taken together, these results suggest that the development of hypertension in an animal model of Type 2 diabetes exacerbates the progression of renal injury, at least in part, by inducing a subacute inflammatory response and that this response can be attenuated with AUDA treatment.

The sEH enzyme has been identified as a target for the treatment of hypertension and inflammation [9]. In animal models of hypertension, sEH inhibitors are reported to decrease BP and thereby inhibit hypertension-induced renal damage [13,18]. The sEH inhibitors CDU and AUDA lowered BP in animal models of hypertension and reduced hypertension-induced renal damage [13]. In animal models of systemic inflammation, sEH inhibitors are reported to decrease the infiltration of pro-inflammatory cells into tissue and decrease the expression of pro-inflammatory genes. The sEH inhibitor AUDA-nBE (AUDA *n*-butyl ester) was reported to inhibit tobacco-smoke-induced lung inflammation [22]. In addition, the sEH inhibitors AUDA-nBE and compound 950 (1-adamantan-3-{5-[2-(2-ethylethoxy)ethoxy])-pentyl}urea) decreased LPS (liposaccharide)-induced increases in plasma concentrations of pro-inflammatory cytokines such TNF-α, IL-6 (interleukin-6) and MCP-5 in mice [23]. In these animal models, the protective effects of the sEH inhibitors are attributed to a decrease in EET metabolism. Although increased epoxides is the probable mechanism, it has been reported that AUDA in addition to sEH inhibition can activate PPAR-α (peroxisome-proliferator-activated receptor-α); however, the concentration of AUDA required for PPAR-α activation is much higher than the concentrations obtained in the present study [29]. In addition, PPAR-α activation would be expected to decrease triacylglycerol levels, which were unchanged in AUDA-treated hypertensive GK rats in the present study. sEH inhibitors have also been reported to increase the incorporation and retention of EETs into endothelial phospholipids and enhance the shuttling of EETs into alternate metabolic pathways [30–32]. The contribution of these alternate actions of sEH inhibitors in the present study are not known and, therefore, might contribute to the renal-protective effects observed.

In the present study, we have demonstrated that hypertension, induced in a model of Type 2 diabetes, exacerbates the development of renal damage. In addition, we provide evidence that this progression involves a subacute inflammatory response. We have demonstrated that sEH inhibition protects the kidney from the development of renal damage, independent of any effects on BP. A possible mechanism by which AUDA treatment attenuates the development of renal damage in this model of hypertension and Type 2 diabetes is via inhibition of the subacute inflammatory response. Taken together, the results provide support for the hypothesis that inhibition of EH ameliorates the inflammatory component of nephropathy associated with hypertension and Type 2 diabetes.

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