

# Antihypertensive action of soluble epoxide hydrolase inhibition in Ren-2 transgenic rats is mediated by suppression of the intrarenal renin–angiotensin system

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## SUMMARY

1. The aim of the present study was to evaluate the hypothesis that the antihypertensive effects of inhibition of soluble epoxide hydrolase (sEH) are mediated by increased intrarenal availability of epoxyeicosatrienoic acids (EETs), with consequent improvement in renal haemodynamic autoregulatory efficiency and the pressure–natriuresis relationship.

2. Ren-2 transgenic rats (TGR), a model of angiotensin (Ang) II-dependent hypertension, and normotensive transgene-negative Hannover Sprague–Dawley (HanSD) rats were treated with the sEH inhibitor *cis*-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy)benzoic acid (c-AUCB; 26 mg/L) for 48 h. Then, the effects on blood pressure (BP), autoregulation of renal blood flow (RBF) and glomerular filtration rate (GFR), and on the pressure–natriuresis relationship in response to stepwise reductions in renal arterial pressure (RAP) were determined.

3. Treatment with c-AUCB did not significantly change BP, renal autoregulation or pressure–natriuresis in normotensive HanSD rats. In contrast, c-AUCB treatment significantly reduced BP, increased intrarenal bioavailability of EETs and significantly suppressed AngII levels in TGR. However, treatment with c-AUCB did not significantly improve the autoregulatory efficiency of RBF and GFR in response to reductions of RAP and to restore the blunted pressure–natriuresis relationship in TGR.

4. Together, the data indicate that the antihypertensive actions of sEH inhibition in TGR are predominantly medi-

ated via significant suppression of intrarenal renin–angiotensin system activity.

**Key words:** cytochrome P450 metabolites, epoxyeicosatrienoic acids, glomerular filtration rate, hypertension, pressure–natriuresis, renal blood flow, renin–angiotensin system, soluble epoxide hydrolase.

## INTRODUCTION

Although the development of hypertension in the Ren-2 renin transgenic rat strain (TGR) is clearly related to the insertion of a mouse Ren-2 renin gene into the genome of normotensive Hannover Sprague–Dawley (HanSD) rats, the exact pathophysiological mechanism(s) responsible for the development and maintenance of hypertension in this monogenetic model of hypertension remains unknown.<sup>1</sup> It has been reported that plasma and kidney angiotensin (Ang) II levels are elevated in conscious heterozygous TGR during the developmental phase of hypertension compared with age-matched HanSD rats.<sup>2–4</sup> In addition, it has been demonstrated that TGR also exhibit enhanced peripheral and renal vascular and tubular responsiveness to AngII compared with HanSD rats.<sup>5,6</sup> Although these findings suggest that the inappropriately activated circulating and intrarenal renin–angiotensin system (RAS) is a critical mechanism responsible for the development of hypertension in this model, recent findings have indicated that the disturbed interaction of the RAS with other vasoactive systems, rather than isolated activation of the RAS, may play a crucial role in the pathophysiology of hypertension in this model.<sup>7–10</sup>

According to this concept, particular attention has been focused on cytochrome P450 (CYP)-dependent metabolites, including epoxyeicosatrienoic acids (EETs), because increasing evidence indicates that lipid mediators play an important role in the regulation of renal tubular ion transport and renal and systemic vascular tone.<sup>11–13</sup> It has been proposed that EETs serve as a compensatory system with protective effects against enhanced RAS activity.<sup>13–15</sup> In addition, it has been demonstrated recently that two kidney, one clip (2K1C) Goldblatt hypertensive rats and

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Cyp1a1-Ren-2 transgenic rats,<sup>16–18</sup> two different models of AngII-dependent hypertension that depend on the enhanced endogenous activity of the RAS, exhibit reduced intrarenal availability of EETs. This is due to increased conversion of EETs to biologically inactive dihydroxyecosatrienoic acids (DHETEs) as a result of increased soluble epoxide hydrolase (sEH) activity.<sup>16–18</sup> Moreover, it has been shown that chronic pharmacological blockade of sEH in these models results in an antihypertensive action that is associated with increased intrarenal availability of EETs, as well as improvements in renal haemodynamic autoregulatory efficiency and the pressure–natriuresis relationship.<sup>17,18</sup> Because TGR reveal a well-documented impairment of the autoregulation of renal haemodynamic and pressure–natriuresis relationship,<sup>19,20</sup> and in view of our recent finding that TGR exhibit reduced tissue availability of EETs,<sup>21</sup> we hypothesized that inhibition of sEH may result in an antihypertensive action that is mediated by an increase in EET bioavailability, with consequent improvement of both renal haemodynamic autoregulation and the impaired pressure–natriuresis relationship.

To test this hypothesis, we evaluated the effects of treatment with the sEH inhibitor *cis*-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy)benzoic acid (c-AUCB) on blood pressure (BP) and on the autoregulation of renal haemodynamic and the pressure–natriuresis relationship in adult TGR with established hypertension.

Furthermore, to gain a more detailed insight into the role of intrarenal interactions of CYP-derived metabolites with the RAS in the regulation of renal function, we determined the renal concentrations of AngII, EETs, DHETEs and 20-hydroxyecosatrienoic acid (20-HETE) in untreated and c-AUCB-treated TGR and HanSD rats. In addition, protein expression of sEH and CYP2C3, the enzyme that is predominantly responsible for the formation of EETs in the kidney, was assessed.

## Methods

### Ethics approval and animals

Studies were performed in accordance with guidelines and practices established by the Animal Care and Use Committee of the Institute for Clinical and Experimental Medicine, Prague, which are in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All animals used in the present study were bred at the Center of Experimental Medicine of this Institute from stock animals supplied by the Max Delbrück Center for Molecular Medicine, Berlin, Germany, which is accredited by the Czech Association for Accreditation of Laboratory Animal Care. Heterozygous TGR were generated by breeding male homozygous TGR with female homozygous HanSD rats, as described and justified in the original study,<sup>1</sup> with age-matched HanSD rats serving as transgene-negative normotensive controls. Rats were kept on a 12 h light–dark cycle. Throughout the experiments, rats were fed a normal-salt, normal-protein diet (0.45% NaCl, 19–21% protein) produced by SEMED (Prague, Czech Republic) and had free access to tap water.

### Chemicals

Rats were treated with sEH inhibitor c-AUCB in drinking water at a dose of 26 mg/L, prepared fresh daily, as described previ-

ously.<sup>16–18</sup> Briefly, crystalline c-AUCB (26 mg) was dissolved in ethanol (5 mL) and (2-hydroxypropyl)- $\beta$ -cyclodextrin (150 mg) after 5 min sonication and this solution was added to 1 L tap water. Hydrogen carbonate sodium (3 mL/L) was given to ensure that the water did not become acidic because low pH can cause the compound to precipitate. This dose of c-AUCB was used in recent studies and exhibited maximal antihypertensive action, as well as substantially increasing tissue concentrations of EETs.<sup>16–18</sup> Untreated control rats were given cyclodextrin and hydrogencarbonate (vehicle) in tap water to drink.

### Experimental design

#### *Series 1: Renal blood flow, glomerular filtration rate and renal sodium excretion responses to decreases in renal arterial pressure*

In this series, heterozygous male TGR with established hypertension (85–90 days of age) and age-matched male HanSD rats were used. Rats were treated with c-AUCB for 48 h because our recent studies have shown that 48 h treatment with c-AUCB elicits the peak decrease in BP in AngII-dependent models of hypertension.<sup>16–18</sup> Thereafter, rats were anaesthetized with sodium thiopental (60 mg/kg, i.p.) and placed on a thermoregulated surgical table to maintain body temperature at 37°C. A tracheostomy was performed and a PE-240 tube was inserted to maintain a patent airway. The exterior end of the tracheal cannula was placed inside a small plastic chamber through which a humidified 95% oxygen/5% carbon dioxide mixture was passed continuously, which has been shown to improve the stability of arterial BP of barbiturate-anaesthetized rats.<sup>22</sup> In this regard, it is important to note that even if barbiturate anaesthesia has some negative effects on BP, we have found in previous studies that the values obtained in anaesthetized rats are an accurate reflection of BP values in conscious rats in which BP was measured by tail plethysmography.<sup>2,6,9</sup> The right jugular vein was catheterized for fluid infusion and anaesthetic administration as needed. The left femoral artery was catheterized with a PE-50 catheter to allow continuous monitoring of arterial BP and blood sampling. Mean arterial pressure (MAP) was monitored with a pressure transducer (model MLT 1050; ADInstruments, Hastings, UK) and recorded on a computer using a computerized data-acquisition system (Power Laboratory/4SP; ADInstruments). The left kidney was exposed via a flank incision, isolated from the surrounding tissue and placed in a lucite cup. The ureter was then cannulated with a PE-10 catheter. An aortic clamp was placed on the aorta above the junction of the left renal artery to regulate the level of renal arterial pressure (RAP). In addition, an ultrasonic transient-time flow probe (1RB; Transonic Systems, Altron Medical Electronic, Fuerstenfeldbruck, Germany) connected to a Transonic flowmeter was placed around the left renal artery and renal blood flow (RBF) was recorded using a computerized data-acquisition system. At the end of the experiment, a zero value was established by complete occlusion of the aorta. During surgery, an isotonic saline solution containing bovine serum albumin (6%; Sigma Chemical; Prague, Czech Republic) was infused at a rate of 40  $\mu$ L/min. After surgery, isotonic saline solution containing albumin (1%) and polyfructosan inulin (7.5%; Inutest; Laevosan, Linz, Austria) was infused at the same infusion rate. After

completion of the surgical procedures, an equilibration period of 50 min was allowed for rats to reach steady state before initiating one 30 min control urine collection at physiological levels of RAP. In groups in which the control protocol was applied, three additional 30 min urine collection periods at physiological levels of RAP were performed. In groups in which the experimental protocol was applied, the first 30 min urine collection was performed at physiological levels of RAP and, thereafter, the 30 min urine collections were performed at reduced RAP of 105, 90 and 80 mmHg. A 5 min equilibration period was allowed after each step reduction in RAP. Blood samples were collected after the second and fourth urine collections to allow determination of glomerular filtration rate (GFR) and renal sodium excretion. This experimental procedure is identical to that used by Wang *et al.*<sup>23</sup> in AngII-infused hypertensive rats and that recently used in our laboratory in Cyp11a1-Ren-2 transgenic rats and 2K1C hypertensive rats.<sup>17,18,24</sup>

Urine volume was measured gravimetrically. Urinary sodium concentrations were determined by flame photometry. Polyfructosan in plasma and urine was measured colorimetrically. Polyfructosan clearance was used as an estimate of GFR. Values were calculated per gram of kidney weight. Fractional sodium excretion ( $FE_{Na}$ ) was calculated using a standard formula. The autoregulatory index (AI) of RBF was calculated according to the method of Semple and de Wardener<sup>25</sup> using the following formula:

$$AI = [(RBF_2 - RBF_1)/RBF_1] / [(RAP_2 - RAP_1)/RAP_1]$$

where  $RBF_1$  and  $RAP_1$  mean renal blood flow and renal arterial pressure before pressure is changed, and  $RBF_2$  and  $RAP_2$  are flow and pressure after the pressure reduction. The same formula was used to calculate the AI of GFR, except that  $RBF_1$  and  $RBF_2$  were replaced with  $GFR_1$  and  $GFR_2$  respectively. An AI of zero indicates ideal autoregulation, whereas higher values indicate impairment of autoregulation.

Rats were divided into the following eight experimental groups: (i) HanSD rats + vehicle + control protocol ( $n = 10$ ); (ii) HanSD rats + c-AUCB + control protocol ( $n = 9$ ); (iii) HanSD rats + vehicle + experimental protocol ( $n = 10$ ); (iv) HanSD rats + c-AUCB + experimental protocol ( $n = 11$ ); (v) TGR + vehicle + control protocol ( $n = 9$ ); (vi) TGR + c-AUCB + control protocol ( $n = 9$ ); (vii) TGR + vehicle + experimental protocol ( $n = 12$ ); and (viii) TGR + c-AUCB + experimental protocol ( $n = 12$ ).

#### Series 2: Assessment of AngII, EETs, DHETEs and 20-HETE concentrations and western blot analysis of renal cortical CYP2C23 and sEH protein expression

Rats were divided into the following four experimental groups and were exposed to the same treatment protocols as in Series 1: (i) HanSD rats + vehicle ( $n = 8$ ); (ii) HanSD rats + c-AUCB ( $n = 8$ ); (iii) TGR + vehicle ( $n = 9$ ); and (iv) TGR + c-AUCB ( $n = 10$ ).

Because it is now well-recognized that plasma and tissue AngII concentrations are higher in anaesthetized rats than in conscious rats that have been decapitated and that normotensive animals exhibit greater increases in renin secretion in response to anaesthesia and surgery than AngII-dependent hypertensive intrarenal

renin-depleted animals,<sup>2,26</sup> in the present study rats from each experimental group were killed by decapitation before collection of plasma and tissue samples. Plasma and whole-kidney AngII levels were measured by radioimmunoassay, as described previously.<sup>2,26</sup> This approach is routinely used in our laboratory and, as such, allows comparisons of results from the present study with those of previous studies to evaluate the role of the RAS in the pathophysiology of hypertension and tissue damage.<sup>2,3,6,8,9,15,16</sup> Levels of the CYP metabolites EETs, DHETEs and 20-HETE were measured in the kidney cortex. Samples were extracted, separated by reverse-phase HPLC and analysed by negative-mode electrospray ionization and tandem mass spectrometry, as described previously.<sup>16,21</sup> Specifically, 8,9-EET, 11,12-EET and 14,15-EET and dihydroxyecosatrienoic acids (DHETEs) were measured separately and were then pooled for clear presentation. Thus, the data are shown as total concentrations of EETs and DHETEs because it is well recognized that these metabolites are the most biologically active products formed in the CYP epoxygenase enzymatic pathway.<sup>13</sup> Western blot analyses for protein expression of CYP2C23 and sEH in the renal cortex were performed as described previously,<sup>16,27</sup> with levels normalized against  $\beta$ -actin.

#### Statistical analyses

All data are expressed as the mean  $\pm$  SEM. Statistical analyses were performed using GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA). The significance of differences were evaluated by the Student's *t*-test, Wilcoxon's signed-rank test for unpaired data or one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test, as appropriate. Repeated-measure ANOVA followed by the Tukey–Kramer multiple comparisons test was used for the analyses within groups (e.g. for the analysis of the autoregulation capacity of RBF and GFR). Values exceeding the 95% probability limits (i.e.  $P < 0.05$ , two-sided) were considered significant.

## RESULTS

### Series 1: RBF, GFR and renal sodium excretion responses to stepwise decreases in RAP

Basal values (average values obtained from the first clearance periods performed at a physiological level of RAP were pooled from groups subjected to the control and experimental protocols) of MAP, bodyweight, RBF, GFR, urine flow, absolute sodium excretion and  $FE_{Na}$  are summarized in Table 1.

As shown in Figs 1a and 2a, HanSD rats maintained autoregulatory efficiency of RBF and GFR in response to reductions of RAP and only the reduction to the lowest level of RAP (80 mmHg) elicited a significant decrease in GFR from  $1.71 \pm 0.11$  to  $0.90 \pm 0.08$  mL/min per g ( $P < 0.05$ ); treatment with c-AUCB did not significantly change the autoregulatory efficiency of RBF or GFR in HanSD rats. As shown in Figs 1b and 2b, untreated TGR exhibited impaired autoregulatory efficiency of RBF and GFR compared with HanSD rats. The reduction of RAP to 80 mmHg elicited significant decreases in RBF in untreated TGR from  $7.45 \pm 0.47$  to  $4.69 \pm 0.37$  mL/min per g

**Table 1** Pooled basal values of mean arterial pressure, renal function and electrolyte excretion in the different groups

	HanSD untreated	HanSD c-AUCB	TGR untreated	TGR c-AUCB
Bodyweight (g)	327 ± 10	332 ± 9	340 ± 11	345 ± 12
MAP (mmHg)	110 ± 3	113 ± 2	149 ± 3 <sup>†</sup>	123 ± 3*
GFR (mL/min per g)	1.61 ± 0.17	1.83 ± 0.19	1.93 ± 0.27	1.85 ± 0.14
RBF (mL/min per g)	6.18 ± 0.22	5.91 ± 0.45	7.42 ± 0.62	7.39 ± 0.61
U <sub>Na</sub> V (μmol/min per g)	1.09 ± 0.37	0.98 ± 0.35	1.75 ± 0.36	0.54 ± 0.09*
FE <sub>Na</sub> (%)	0.91 ± 0.26	0.84 ± 0.22	1.27 ± 0.26	0.49 ± 0.08*
Urine flow (μL/min per g)	11.86 ± 2.94	9.98 ± 2.65	18.42 ± 2.42*	9.12 ± 1.49

Data are the mean ± SEM. \* $P < 0.05$  compared with all unmarked values; <sup>†</sup> $P < 0.05$  compared with all values (one-way ANOVA followed by the Tukey–Kramer multiple comparisons test).

HanSD, transgene-negative Hannover Sprague–Dawley rats; TGR, Ren-2 transgenic rats; c-AUCB, treatment with the soluble epoxide hydrolase inhibitor; MAP, mean arterial pressure; GFR, glomerular filtration rate; RBF, renal blood flow; U<sub>Na</sub>V, absolute sodium excretion; FE<sub>Na</sub>, fractional sodium excretion.

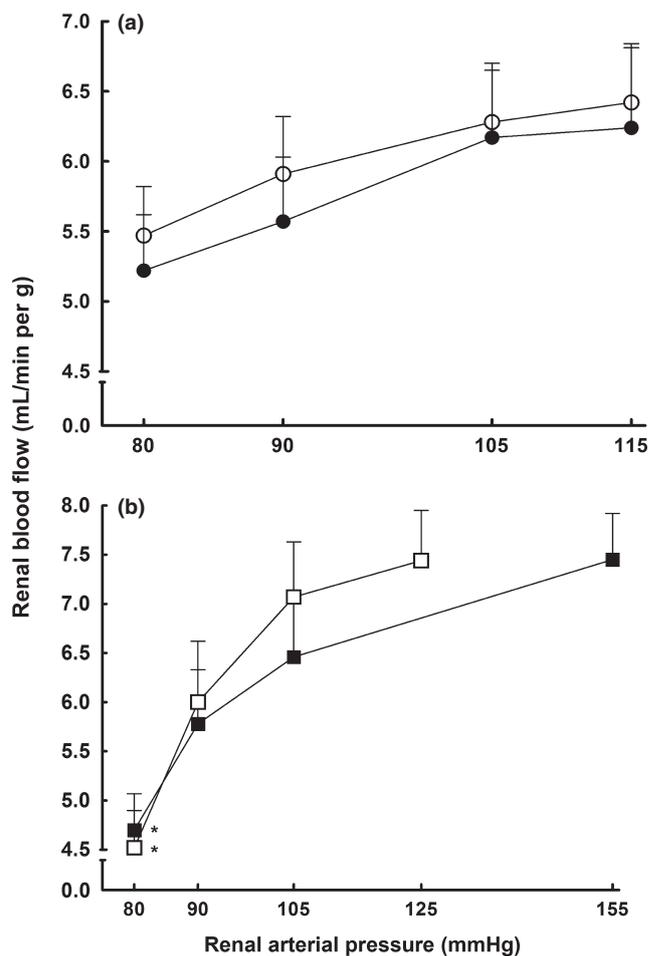
( $P < 0.05$ ), with significant decreases in GFR already observed at the level of RAP of 105 mmHg in untreated TGR. Treatment with c-AUCB did not significantly improve the autoregulatory efficiency of RBF and GFR in TGR.

Figure 3 shows the autoregulatory efficiency of RBF and GFR using AI calculated according to the method of Semple and de Wardener.<sup>25</sup> Results of AI calculations show that untreated TGR already exhibited substantial impairment of the autoregulatory efficiency of RBF and GFR following reduction of RAP from 105 to 90 mmHg. Treatment with c-AUCB did not restore the AI of RBF and GFR in TGR.

As shown in Figs 4a and 5a, treatment with c-AUCB had no significant effect on urine flow, absolute sodium excretion or FE<sub>Na</sub> (data not shown) at the physiological levels of RAP in HanSD rats. Reducing RAP elicited similar responses in both groups of HanSD rats.

As shown in Figs 4b and 5b, reducing RAP resulted in significantly greater decreases in urine flow and absolute sodium excretion in untreated TGR compared with HanSD rats; the differences were significant at RAP of 105 mmHg and were further pronounced at RAP of 90 and 80 mmHg. Treatment of TGR with c-AUCB had no significant effect on the responses of urine flow and absolute sodium excretion to reductions in RAP in TGR.

Untreated and c-AUCB-treated TGR and HanSD rats subjected to the control protocol did not exhibit any significant changes in renal haemodynamics or renal sodium excretion throughout the experiment and therefore the data are not presented.

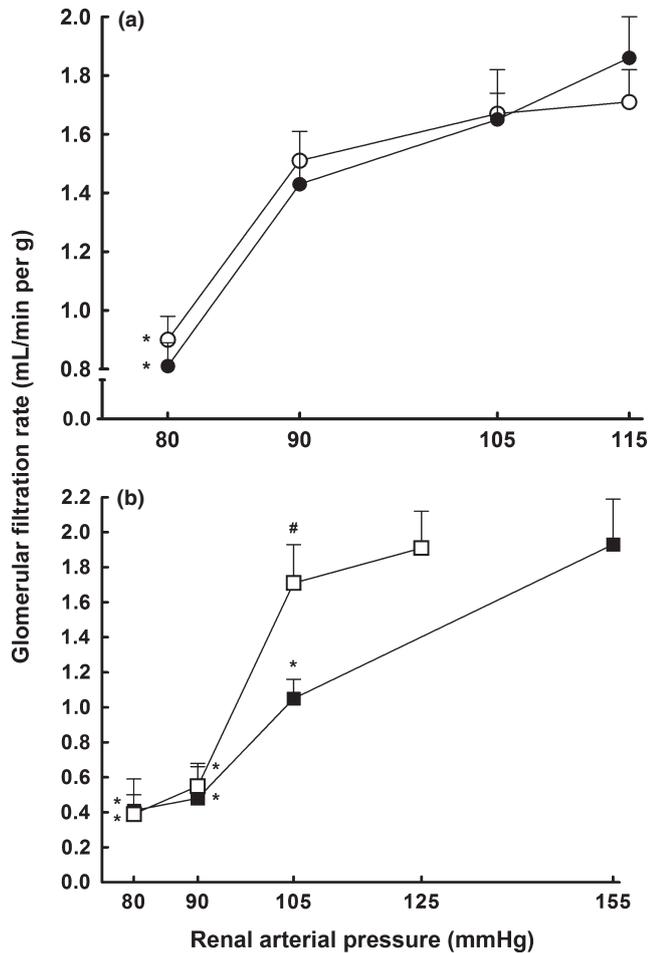


**Fig. 1** Relationship between renal arterial pressure and renal blood flow in (a) transgene-negative Hannover Sprague–Dawley (HanSD) rats and (b) Ren-2 transgenic rats (TGR) treated with or without *cis*-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy)benzoic acid (c-AUCB). (○), untreated HanSD; (●), c-AUCB-treated HanSD; (■), untreated TGR; (□), c-AUCB-treated TGR. Data are the mean ± SEM. \* $P < 0.05$  compared with basal values (repeated-measures ANOVA followed by the Tukey–Kramer multiple comparisons test).

### Series 2: Assessment of AngII, EETs, DHETEs and 20-HETE concentrations and western blot analysis for renal cortical CYP2C23 and sEH protein expression

Densitometric analysis revealed that, when normalized for  $\beta$ -actin, there were no significant differences in CYP2C3 or sEH protein expression in the renal cortex between TGR and HanSD rats with or without c-AUCB treatment. In addition, there were no significant differences in renal concentrations of 20-HETE between untreated TGR and untreated HanSD rats ( $4098 \pm 198$  vs  $4127 \pm 322$  ng/g protein) and treatment with c-AUCB did not significantly change 20-HETE concentrations in either TGR or HanSD rats.

Plasma AngII levels were higher in untreated TGR than in untreated HanSD rats ( $30 \pm 2$  vs  $9 \pm 1$  fmol/mL;  $P < 0.05$ ). Treatment with c-AUCB did not significantly change plasma AngII levels in HanSD rats, but significantly decreased them in TGR (to  $18 \pm 1$  fmol/mL;  $P < 0.05$ ). As shown in Fig. 6a,



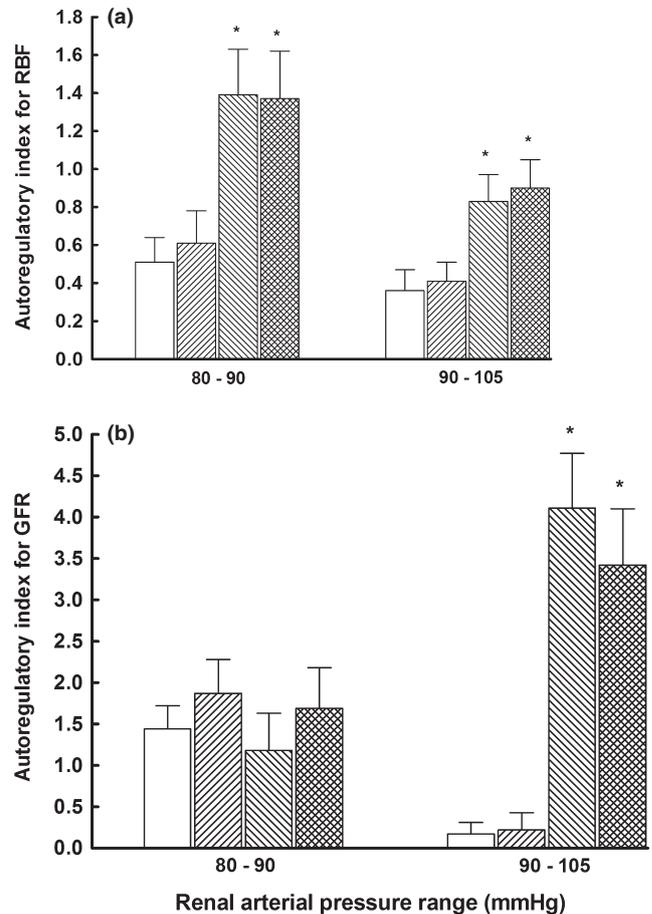
**Fig. 2** Relationship between renal arterial pressure and glomerular filtration rate in (a) transgene-negative Hannover Sprague–Dawley (HanSD) rats and (b) Ren-2 transgenic rats (TGR) treated with or without *cis*-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy)benzoic acid (c-AUCB). (○), untreated HanSD; (●), c-AUCB-treated HanSD; (■), untreated TGR; (□), c-AUCB-treated TGR. Data are the mean  $\pm$  SEM. \* $P < 0.05$  compared with basal values (repeated-measures ANOVA followed by the Tukey–Kramer multiple comparisons test). # $P < 0.05$  compared with corresponding values from untreated rats (unpaired Student's *t*-test).

kidney AngII levels were significantly higher in untreated TGR than in untreated HanSD rats ( $38 \pm 4$  vs  $23 \pm 2$  fmol/g tissue;  $P < 0.05$ ). Treatment with c-AUCB significantly decreased kidney AngII levels in TGR, but not in HanSD rats.

As shown in Fig. 6b, the intrarenal availability of biologically active epoxygenase metabolites (when expressed as the EETs/DHETEs ratio) was significantly lower in untreated TGR than untreated HanSD rats ( $2.16 \pm 0.29$  vs  $3.14 \pm 0.23$ ;  $P < 0.05$ ). Treatment with c-AUCB significantly increased this ratio in both TGR and HanSD rats compared with basal values ( $P < 0.05$  for both).

## DISCUSSION

The results of the present study demonstrate that c-AUCB treatment significantly reduces BP in TGR and is associated with significant increases in the availability of biologically active epoxygenase metabolites, as evidenced by the EETs/DHETEs ratio. However, in contrast with our hypothesis, these actions were not

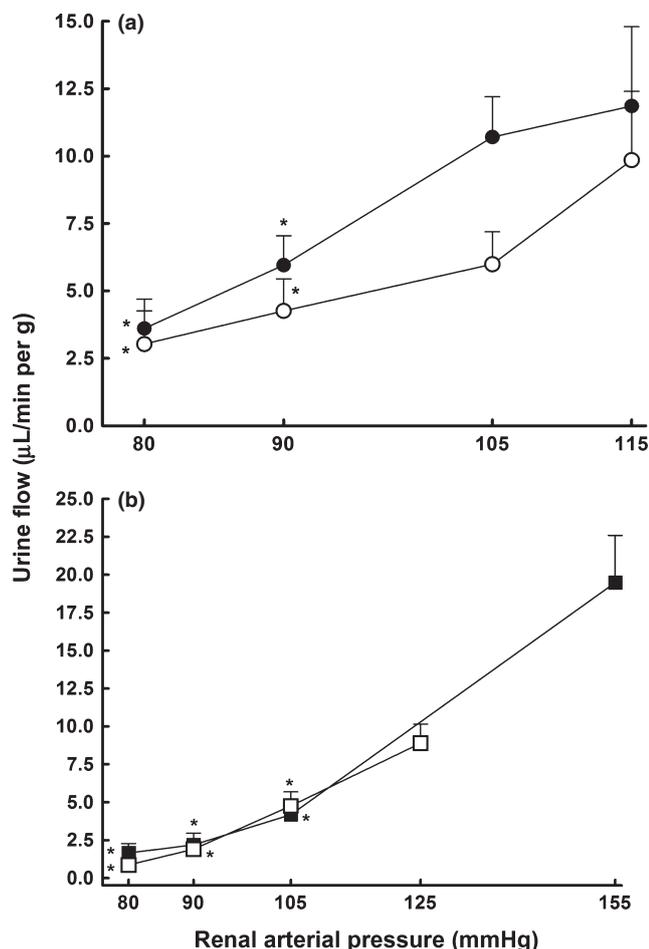


**Fig. 3** Autoregulatory indices calculated according to the method of Semple and de Wardener<sup>25</sup> for (a) renal blood flow (RBF) and (b) glomerular filtration rate (GFR) responses to reductions in renal arterial pressure from 105 to 90 mmHg and from 90 to 80 mmHg in transgene-negative Hannover Sprague–Dawley (HanSD) rats and Ren-2 transgenic rats (TGR) treated with or without *cis*-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy)benzoic acid (c-AUCB). (□), untreated HanSD; (□), c-AUCB-treated HanSD; (▨), untreated TGR; (▩), c-AUCB-treated TGR. Data are the mean  $\pm$  SEM. \* $P < 0.05$  compared with unmarked values at the same level of renal arterial pressure (one-way ANOVA followed by the Tukey–Kramer multiple comparisons test).

accompanied by an improvement in autoregulatory efficiency of RBF and GFR or the slope of the pressure–natriuresis relationship. In addition, to our surprise, treatment with c-AUCB resulted in a significant reduction in plasma AngII levels and normalization of kidney AngII concentrations in TGR to levels observed in untreated HanSD rats. Moreover, our data show that c-AUCB treatment does not alter AngII level in HanSD rats.

Because the results of the present study strongly oppose our hypothesis that the antihypertensive action of c-AUCB treatment is due to EETs-mediated improvements in the autoregulatory efficiency of renal haemodynamics and of the blunted pressure–natriuresis relationship in TGR, the critically important issue of the present study is related to the following question: what is the mechanism(s) responsible for the BP-lowering effect of c-AUCB treatment in TGR?

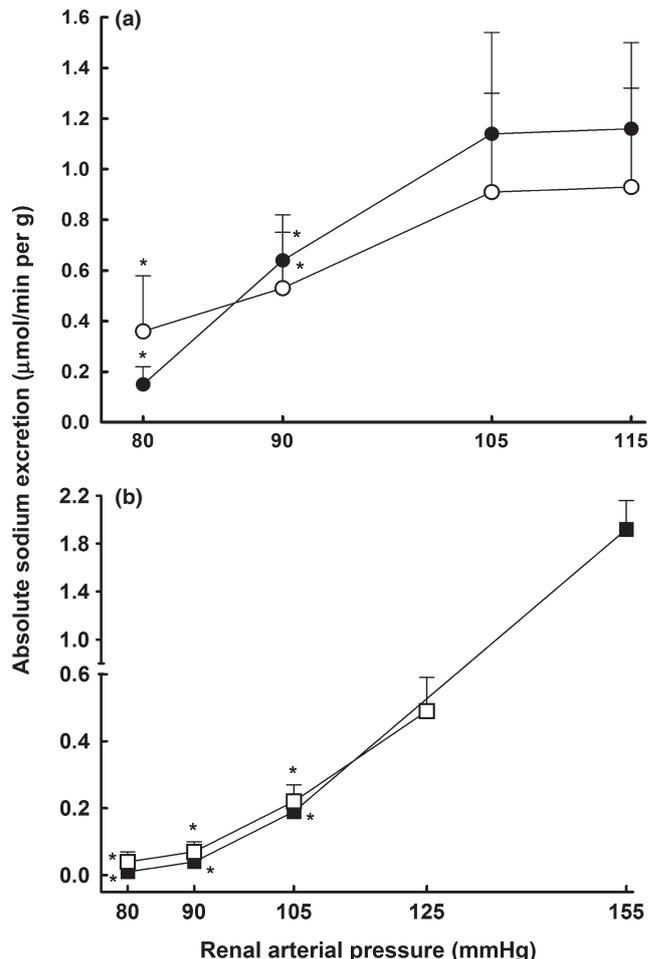
In this regard, it is important to realise that studies performed during the past three decades have revealed that EETs exert important biological actions on the regulation of vascular tone



**Fig. 4** Relationship between renal arterial pressure and urine flow in (a) transgene-negative Hannover Sprague-Dawley (HanSD) rats and (b) Ren-2 transgenic rats (TGR) treated with or without *cis*-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy)benzoic acid (c-AUCB). (●), untreated HanSD; (○), c-AUCB-treated HanSD; (■), untreated TGR; (□), c-AUCB-treated TGR. Data are the mean  $\pm$  SEM. \* $P < 0.05$  compared with basal values (repeated-measures ANOVA followed by the Tukey–Kramer multiple comparisons test).

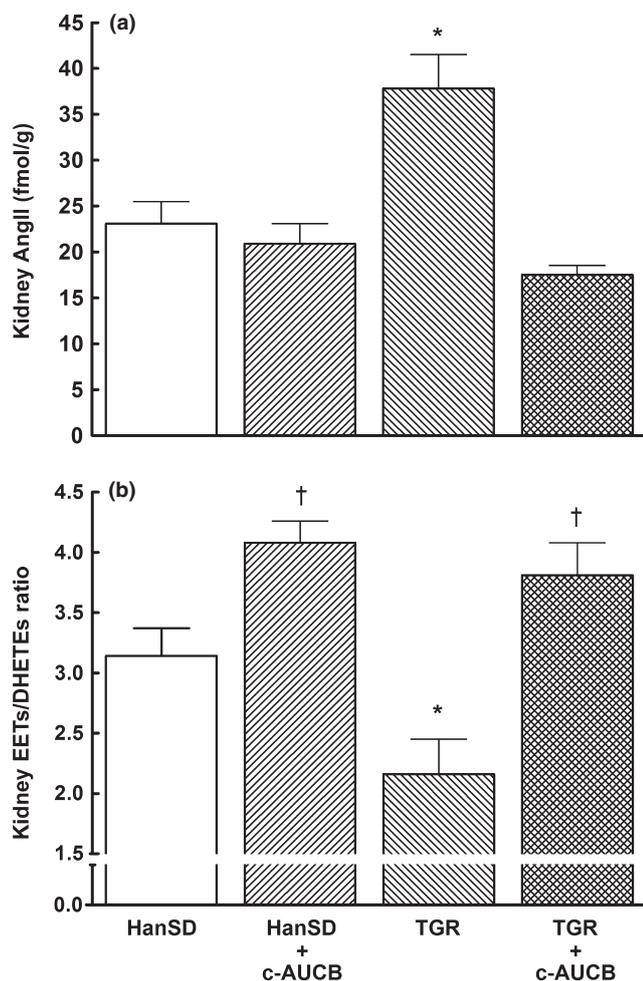
and especially on the control of the renal tubular transport of sodium.<sup>11–13</sup> The EETs have been consistently shown to cause vasodilatation by stimulating large-conductance calcium-activated potassium channels.<sup>11,12,28</sup> They have also been identified as endothelium-derived hyperpolarizing factors that mediate nitric oxide- and prostaglandin-independent vasodilatation and have been found to oppose the vasoconstrictor actions of AngII.<sup>14,15,29–31</sup> On the basis of these results, it is still conceivable that one potential mechanism underlying the BP-lowering action of c-AUCB treatment in TGR could be EETs-mediated attenuation of the previously well-documented selective peripheral and renal vascular responsiveness to AngII.<sup>5,6</sup>

However, most of the evidence available thus far indicates that the antihypertensive properties of EETs are related to their action on renal sodium excretion<sup>12,13</sup> because, at the level of the kidney, it has been shown that EETs inhibit sodium reabsorption in the proximal tubule by blocking the sodium–hydrogen exchanger<sup>32</sup> and also decrease sodium reabsorption in the cortical collecting duct by blocking epithelial sodium channels.<sup>33</sup> In addition, it has



**Fig. 5** Relationship between renal arterial pressure and absolute sodium excretion in (a) transgene-negative Hannover Sprague-Dawley (HanSD) rats and (b) Ren-2 transgenic rats (TGR) treated with or without *cis*-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy)benzoic acid (c-AUCB). (●), untreated HanSD; (○), c-AUCB-treated HanSD; (■), untreated TGR; (□), c-AUCB-treated TGR. Data are the mean  $\pm$  SEM. \* $P < 0.05$  compared with basal values (repeated-measures ANOVA followed by the Tukey–Kramer multiple comparisons test).

been demonstrated that EETs play an important role in the regulation of the afferent arteriole autoregulatory responses to changes in perfusion pressure by limiting pressure-mediated vasoconstriction.<sup>34</sup> It was therefore conceivable to assume that net intrarenal deficiency of EETs in TGR contributes to the well-known impairment of the pressure–natriuresis relationship<sup>19,20</sup> and, in accordance with the concept originally proposed by Guyton *et al.*<sup>35</sup> and supported by findings from several other groups,<sup>23,36–39</sup> this impairment is the critical mechanism responsible for the pathophysiology of hypertension. This notion has been further supported by our recent findings that chronic inhibition of sEH normalized the intrarenal bioavailability of EETs and improved the pressure–natriuresis relationship in 2K1C Goldblatt hypertensive and Cyp11a1-Ren-2 transgenic rats and was associated with significant BP-lowering effects.<sup>17,18</sup> However, the results of the present study clearly indicate that this is not the underlying mechanism of the BP-lowering actions of c-AUCB treatment in TGR. We cannot offer a fully satisfactory explanation as to why



**Fig. 6** Kidney (a) angiotensin (Ang) II levels and (b) the epoxyeicosatrienoic acids (EETs)/dihydroxyeicosatrienoic acids (DHETEs) ratio in (a) transgene-negative Hannover Sprague–Dawley (HanSD) rats and (b) Ren-2 transgenic rats (TGR) treated with or without *cis*-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy)benzoic acid (c-AUCB). Data are the mean  $\pm$  SEM. \* $P < 0.05$  compared with unmarked values; † $P < 0.05$  compared with all other values (one-way ANOVA followed by the Tukey–Kramer multiple comparisons test).

in two other different models of AngII-dependent hypertension, which similarly depend on the enhanced endogenous activity of the RAS, the actions of EETs at the kidney level were responsible for the antihypertensive actions of the same sEH inhibitor (c-AUCB), which, in addition, was used at the same dose.

Nevertheless, treatment of TGR with c-AUCB markedly decreased plasma AngII and normalized intrarenal AngII to levels seen in HanSD rats. In view of our findings it seems reasonable to assume that suppression of AngII levels is the main underlying mechanism responsible for the BP-lowering action of c-AUCB treatment. This notion is in good agreement with previous studies that have shown that inappropriate activation of the intrarenal RAS is the main contributor to the pathophysiology of the AngII-dependent form of hypertension.<sup>40</sup> In addition, the findings of the present study are in agreement with studies showing that only chronic, and not acute, blockade of the RAS restores the pressure–natriuresis relationship, indicating long-term modulatory

actions of elevated AngII levels on the slope of the pressure–natriuresis relationship and suggesting that the acute BP-lowering effects of RAS inhibition are primarily attributable to changes in total peripheral resistance.<sup>23,41</sup> Furthermore, this notion is supported by a recent study in homozygous and heterozygous TGR<sup>42</sup> that showed that acute RAS inhibition (by acute intravenous administration of the angiotensin-converting enzyme inhibitor captopril) resulted in acute, profound decreases in BP that were predominantly mediated by a decrease in total peripheral resistance. However, it is difficult to reconcile the present findings in TGR with recent findings in AngII-dependent models of hypertension that show that c-AUCB treatment (at the same dose) does not alter circulating and renal RAS activity.<sup>16–18</sup> However, one possible explanation may be offered: Heinrich *et al.*<sup>43</sup> have found that 14,15-EET does not alter basal renin release, but exhibits a marked inhibitory effect on renin release stimulated by isoproterenol in renal cortical slices. It is therefore conceivable that enhanced intrarenal tissue availability of EETs could suppress the activity of the mouse Ren-2 renin gene, which could lead to suppression of AngII concentrations in TGR without altering AngII levels in transgene-negative HanSD rats.

Nevertheless, it is important to recognize that Bohlender *et al.*<sup>44</sup> have shown that the regulation of both renin concentrations and activity in TGR is extremely complicated because one must consider the mouse as well as rat renin concentrations (and activity) in these TGR. Therefore, it is obvious that additional studies that are beyond the scope of the present study are necessary to address this issue; currently, we cannot offer a fully satisfactory explanation(s) for these apparent discrepancies.

However, in this regard, it is important to recognize that previous studies have demonstrated that increased intrarenal AngII concentrations in AngII-dependent models of hypertension are the result of a combination of enhanced AngII production from endogenous intrarenal components and the uptake of circulating AngII by AT<sub>1</sub> receptors.<sup>40,45</sup> Therefore, it is conceivable that the normalization of intrarenal AngII concentrations in c-AUCB-treated TGR is the consequence EETs-mediated suppression of intrarenal AngII production due to suppression of intrarenal renin activity combined with decreased AT<sub>1</sub> receptor-mediated uptake of AngII from the circulation as the result of decreased plasma AngII. This notion could be fully corroborated by studies using an experimental protocol in which c-AUCB is coadministered with an AT<sub>1</sub> receptor antagonist. In addition, it is well-known that oxidative stress plays an important role in the pathogenesis of AngII-dependent forms of hypertension.<sup>46</sup> Thus, we have recently reported that TGR exhibit increased intrarenal oxidative stress<sup>8</sup> that exerts renal vasoconstrictor and antinatriuretic effects and modulates renal function in prehypertensive TGR.<sup>9</sup> It is therefore possible that normalization of intrarenal AngII concentrations in c-AUCB-treated TGR may reduce increased oxidative stress, which could contribute, at least in part, to the antihypertensive actions of c-AUCB in those rats. However, these issues require further investigation, which is beyond the scope of the present study.

Taking all these observations into consideration, we suggest that the underlying mechanism(s) responsible for the BP-lowering effect of 48 h treatment with the sEH inhibitor c-AUCB in TGR is related to the combination of enhanced EETs bioavailability and their effects at the vascular level, with the marked suppression of RAS activity. We suggest that the main mechanism

underlying the BP-lowering actions of c-AUCB treatment in TGR in the present study is a decrease in peripheral vascular resistance that is mediated by the increased bioavailability of vasodilatory EETs and decreased concentrations of the vasoconstrictor AngII.

In conclusion, the results of the present study indicate that treatment with the sEH inhibitor c-AUCB: (i) significantly reduces BP in TGR and elicits substantial increases in the intrarenal availability of endogenous, biologically active epoxygenase metabolites; (ii) does not improve the autoregulatory efficiency of renal haemodynamics and of the blunted pressure–natriuresis mechanism in TGR; and (iii) results in marked suppression of plasma AngII levels and normalization of renal AngII concentrations in TGR to levels observed in normotensive HanSD rats.

Together, these findings indicate that the antihypertensive action of sEH inhibition by c-AUCB in TGR rats is predominantly mediated by suppression of systemic and intrarenal RAS activity.

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