

Inhibition of soluble epoxide hydrolase improves the impaired pressure–natriuresis relationship and attenuates the development of hypertension and hypertension-associated end-organ damage in Cyp1a1-Ren-2 transgenic rats

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Objective In the present study, we compared the effects of treatment with the novel soluble epoxide hydrolase (sEH) inhibitor (c-AUCB) with those of the AT₁ receptor antagonist losartan on blood pressure (BP), autoregulation of renal blood flow (RBF) and on glomerular filtration rate (GFR) and the pressure–natriuresis relationship in response to stepwise reduction in renal arterial pressure (RAP) in Cyp1a1-Ren-2 transgenic rats.

Methods Hypertension was induced in Cyp1a1-Ren-2 rats through dietary administration for 11 days of the natural xenobiotic indole-3-carbinol (I3C) which activates the renin gene. Treatment with c-AUCB and losartan was started 48 h before initiating administration of the diet containing I3C. Rats were prepared for renal functional studies to evaluate in-vivo renal autoregulatory efficiency when RAP was gradually decreased by an aortic clamp.

Results I3C administration resulted in the development of severe hypertension which was associated with markedly lower basal RBF and GFR and substantially impaired autoregulatory efficiency as well as a suppression of the pressure–natriuresis relationship when compared with noninduced rats. Treatment with c-AUCB significantly decreased BP, improved autoregulatory efficiency of RBF and GFR and the slope of pressure–natriuresis relationship. Treatment with losartan completely prevented the impaired autoregulation and pressure–natriuresis relationship as well as the development of hypertension in I3C-induced rats.

Conclusion Our present findings indicate that chronic treatment with the sEH inhibitor c-AUCB substantially

attenuates the development of malignant hypertension in I3C-induced rats likely via improvement of the renal autoregulatory efficiency and the pressure–natriuresis relationship. *J Hypertens* 29:1590–1601 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: AT₁ receptor antagonist, cytochrome P-450 metabolites, epoxyeicosatrienoic acids, malignant hypertension, renal autoregulation, renin-angiotensin system, sodium excretion, soluble epoxide hydrolase

Abbreviations: ANG II, angiotensin II; AT₁, receptors for angiotensin II* type 1; BP, blood pressure; c-AUCB, cis-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyl-oxy]-benzoic acid; CYP, cytochrome P-450; DHETEs, dihydroxyeicosatrienoic acids; EETs, epoxyeicosatrienoic acids; ENaC, epithelial sodium channel; GFR, glomerular filtration rate; GSI, glomerulosclerosis index; I3C, indole-3-carbinol; LVW, left heart ventricle weight; MAP, mean arterial pressure; RAP, renal arterial pressure; RAS, renin–angiotensin system; RBF, renal blood flow; sEH, soluble epoxide hydrolase

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Introduction

It is well recognized that the renin-angiotensin system (RAS) physiologically plays a central role in the regulation of blood pressure (BP) and it has also been demonstrated that the inappropriate activation of this powerful system critically contributes to the pathophysiology of hypertension [1,2]. Nevertheless, emerging evidence indicates that the role of the RAS in the regulation of BP and the pathophysiology of hypertension is more

complex than so far thought and remains still incompletely understood [1,3,4].

According to the concept originally proposed by Guyton *et al.* [5] and supported by many other groups [2,6,7], the kidney's pressure–natriuresis mechanism plays a dominant role in the long-term BP regulation. It has also been shown that activation of the RAS – when not adequately counteracted by other physiological systems – can lead to

a marked suppression of the pressure–natriuresis relationship which has been implicated in the pathophysiology of hypertension, especially in the model of angiotensin II (ANG II)-dependent hypertension [1,2,8–13]. However, the role of counteracting compensatory mechanisms that dampen the effects of ANG II-induced vasoconstriction under conditions of excessive activation of the RAS remains poorly understood, especially in models of an ANG II-dependent malignant form of hypertension. Methodological limitations related to the mode of induction of this form of hypertension have so far hampered the approach of how to study this issue.

Recently, an inbred transgenic rat line [strain name: TGR(Cyp1a1-Ren-2)] was generated, which offers a new possibility to precisely control the development of an ANG II-dependent malignant form hypertension [14]. The Cyp1a1-Ren-2 transgenic rat was generated by inserting the mouse Ren-2 renin gene, fused to the cytochrome P-450 (Cyp1a1) promoter, into the genome of the Fischer 344 rat. The Cyp1a1 promoter is not constitutively expressed in the liver; however, after exposure to various natural xenobiotics such as indole-3-carbinol (I3C) the expression of the Cyp1a1 promoter is rapidly enhanced with a marked increase of the expression of the Ren-2 renin gene in the liver with a subsequent increase in ANG II levels [14]. We and others [15–18] have demonstrated that the endogenous activity of the RAS and subsequently the degree of BP up to a level of malignant hypertension and associated end-organ damage can be precisely regulated in a dose-dependent and time-dependent way.

Increasing evidence indicates that cytochrome P450 (CYP)-dependent metabolites of arachidonic acid, such as epoxyeicosatrienoic acids (EETs), play an important role in the regulation of renal tubular ion transport and renal and systemic vascular tone and it has been suggested that EETs serve as a compensatory system with protective effects against enhanced RAS activity [19–23]. In addition, results from our recent study have revealed that substantial increases in the intrarenal level of endogenous EETs significantly attenuated the development of malignant hypertension in Cyp1a1-Ren-2 transgenic rats [17]. In view of these findings, we hypothesized that the antihypertensive actions of the inhibition of soluble epoxide hydrolase (sEH) – an enzyme responsible for the conversion of EETs to biologically inactive dihydroxyeicosatrienoic acids (DHETEs) – in this ANG II-dependent model of malignant hypertension are mediated by an improvement of the impaired renal autoregulatory efficiency of the pressure–natriuresis mechanism.

To test this hypothesis, the first aim of the present study was to characterize the effects of chronic treatment with the novel sEH inhibitor *cis*-4-[4-(3-adamantan-1-ylureido)-cyclohexyl-oxy]-benzoic acid (*c*-AUCB) [24] on

the autoregulation of renal hemodynamics, the pressure–natriuresis relationship and the development of the associated hypertension-induced cardiac hypertrophy and renal injury after induction of the renin gene in Cyp1a1-Ren-2 transgenic rats.

As recent studies have demonstrated that activation of ANG II type 1 receptors (AT₁) contributes to the augmented BP and the impairment of renal function in Cyp1a1-Ren-2 transgenic rats with malignant hypertension [15,25], the second aim of the present study was to compare the effects of chronic AT₁ receptor blockade with those of chronic sEH inhibition on BP, renal function and hypertension-associated end-organ damage in this model of ANG II-dependent malignant hypertension.

Methods

The studies were performed in accordance with guidelines and practices established by the Institute for Clinical and Experimental Medicine Animal Care and Use Committee and are in accordance with laws in the Czech Republic. All animals used in the present study were housed in facilities accredited by the Czech Association for Accreditation of Laboratory Animal Care.

Animals and diets

Please see the online Data Supplement, <http://links.lww.com/HJH/A98> for details.

Experimental design

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

To induce malignant hypertension, Cyp1a1-Ren-2 rats were fed a diet containing 0.3% I3C for 11 days, which results in the development of malignant hypertension with markedly elevated circulating and tissue ANG II levels as we and others [14–18] have demonstrated. The sEH inhibitor *c*-AUCB was given at a dose of 26 mg/l in drinking water that was prepared freshly every third day as described previously [17]. Treatment with *c*-AUCB was started 48 h before feeding the diet with or without I3C. This dose of *c*-AUCB and the same treatment protocol for *c*-AUCB were used in our recent study in which we found that it significantly attenuated the development of malignant hypertension in Cyp1a1-Ren-2 transgenic rats and substantially increased tissue concentrations of EETs [17]. Animals who were exposed to AT₁ receptor blockade received losartan in their drinking water (100 mg/l; Lozap, Zentiva, Prague, Czech Republic); previous studies have demonstrated that this dose of losartan prevents the development of hypertension in this model [25,26]. On the day of the experiment (day 11 after induction of the renin gene) rats were prepared for acute renal functional studies and for details see the online Data Supplement, <http://links.lww.com/HJH/A98>.

The following experimental groups of Cyp1a1-Ren-2 transgenic rats were examined:

Group 1: noninduced + untreated + control protocol ($n = 6$).

Group 2: noninduced + untreated + experimental protocol ($n = 13$).

Group 3: noninduced + *c*-AUCB + control protocol ($n = 7$).

Group 4: noninduced + *c*-AUCB + experimental protocol ($n = 11$).

Group 5: noninduced + losartan + control protocol ($n = 6$).

Group 6: noninduced + losartan + experimental protocol ($n = 10$).

Group 7: induced + untreated + control protocol ($n = 7$).

Group 8: induced + untreated + experimental protocol ($n = 13$).

Group 9: induced + *c*-AUCB + control protocol ($n = 7$).

Group 10: induced + *c*-AUCB + experimental protocol ($n = 13$).

Group 11: induced + losartan + control protocol ($n = 6$).

Group 12: induced + losartan + experimental protocol ($n = 11$).

Series 2: effects of chronic soluble epoxide hydrolase inhibition and AT₁ receptor blockade on cardiac hypertrophy, proteinuria, renal glomerular damage and kidney tubulointerstitial injury as well as concentrations of epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids in renal tissue

Animals were divided into the following experimental groups and were exposed to the same chronic experimental protocol as in series 1:

Group 1: noninduced + untreated ($n = 7$).

Group 2: noninduced + *c*-AUCB ($n = 6$).

Group 3: noninduced + losartan ($n = 6$).

Group 4: induced + untreated ($n = 8$).

Group 5: induced + *c*-AUCB ($n = 8$).

Group 6: induced + losartan ($n = 8$).

Urine collections were performed prior (72 h before) and 10 days after initiating the administration of the diet with or without I3C to assess daily proteinuria as described previously [17]. On day 11, rats were sacrificed by decapitation and always one kidney was used for evaluation of renal glomerular damage and renal cortical tubulointerstitial injury using the following method, which is now routinely employed in our laboratory [27,28] and for

details see the online Data Supplement, <http://links.lww.com/HJH/A98>.

The level of the arachidonic acid metabolites, EETs and DHETEs, were measured in the kidney cortex. Samples were extracted, separated by reverse-phase, high-performance liquid chromatography, and analyzed by negative-mode electrospray ionization and tandem mass spectroscopy as described previously [17].

Statistical analyses

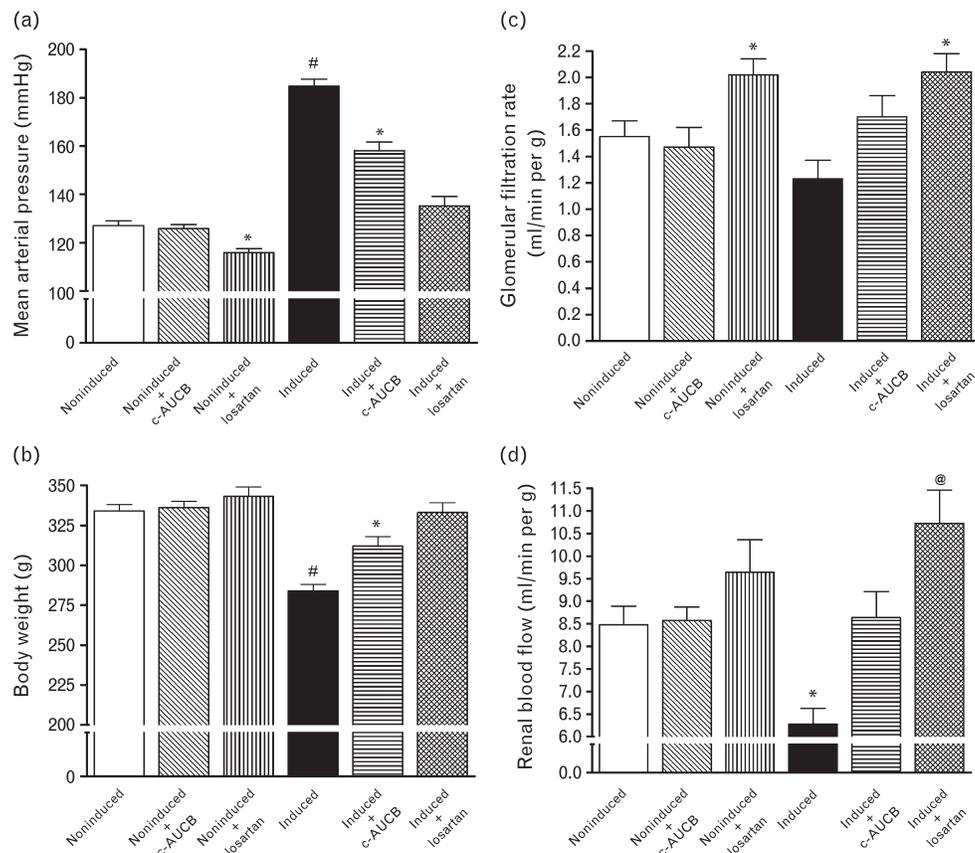
All values are expressed as means \pm SEM. With GraphPad Prism software (Graph Pad Software, San Diego, California, USA), statistical analysis was performed using Student's *t*-test, Wilcoxon's signed-rank test for unpaired data, or one-way analysis of variance (ANOVA) when appropriate. ANOVA for repeated measurements, followed by Student–Newman–Keuls test was performed for the analysis within groups [e.g. for the analysis of autoregulation capacity of renal blood flow (RBF) and glomerular filtration rate (GFR)]. Values exceeding the 95% probability limits ($P < 0.05$) were considered statistically significant.

Results

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

Basal values [average values obtained from the first clearance periods performed at a physiological level of renal arterial pressure (RAP) were pooled from groups exposed to control and experimental protocols] of mean arterial pressure (MAP), body weight, RBF and GFR are summarized in Fig. 1. Treatment with the sEH inhibitor *c*-AUCB did not alter MAP, body weight, RBF and GFR in noninduced rats when compared with untreated noninduced normotensive Cyp1a1-Ren-2 rats. In contrast, treatment with the AT₁ receptor antagonist losartan significantly decreased MAP and GFR, but did not change body weight and RBF in noninduced normotensive Cyp1a1-Ren-2 rats. Administration of the diet containing 0.3% I3C resulted in severe hypertension (Fig. 1a), which was associated with a profound loss of body weight (Fig. 1b) and a marked reduction in RBF (Fig. 1c) as compared with noninduced animals. In addition, untreated I3C-induced Cyp1a1-Ren-2 rats demonstrated serious lethargy, hunched posture and piloerection, which are manifestations of a malignant form of hypertension in rats [15,18,34]. Treatment with *c*-AUCB attenuated the development of hypertension and the loss in body weight and prevented the decreases in RBF in I3C-induced Cyp1a1-Ren-2 transgenic rats as compared with untreated I3C-induced Cyp1a1-Ren-2 rats. Treatment with losartan abolished the increases in MAP and decreases in body weight and not only prevented the decreases in RBF in I3C-induced Cyp1a1-Ren-2 transgenic rats, but even significantly

Fig. 1



Basal values of mean arterial pressure (a), body weight (b), renal blood flow (d) and glomerular filtration rate (c) in Cyp1a1-Ren-2 transgenic rats fed either a normal rat chow (noninduced) or a diet containing 0.3% indole-3-carbinol (I3C-induced). c-AUCB indicates treatment with the sEH inhibitor c-AUCB, losartan indicates treatment with AT₁ receptor antagonist. * $P < 0.05$ vs. unmarked values. @ $P < 0.05$ vs. untreated noninduced rats. # $P < 0.05$ vs. all values.

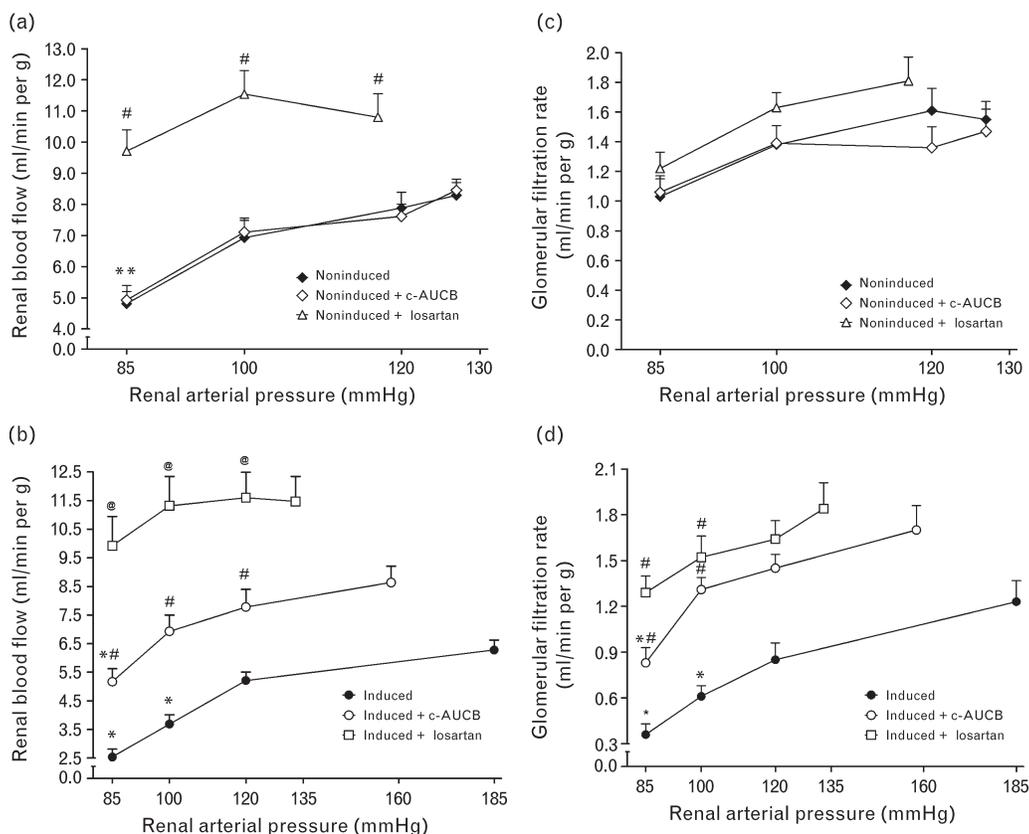
increased it as compared with noninduced normotensive Cyp1a1-Ren-2 rats (Fig. 1d).

As shown in Fig. 2a and c, noninduced rats maintained autoregulatory efficiency of RBF and GFR in response to reductions in RAP without any effect of treatment with c-AUCB. Treatment with losartan did not alter autoregulatory efficiency of GFR in noninduced rats; however, it increased not only basal values of RBF, but as well improved the autoregulation of RBF at the lowest level of RAP ($P < 0.05$) (Fig. 2a). As shown in Fig. 2b at the basal level of RAP RBF was significantly lower in untreated I3C-induced Cyp1a1-Ren-2 rats as compared with noninduced animals (6.29 ± 0.35 vs. 8.29 ± 0.41 ml/min per g, $P < 0.05$) and reduction of RAP resulted in significant decreases in RBF already at a level of RAP of 100 mmHg. In contrast, the treatment with c-AUCB in I3C-induced Cyp1a1-Ren-2 transgenic rats not only prevented the decrease in RBF at the basal level of RAP, but also markedly improved the autoregulatory efficiency of RBF to levels

observed in noninduced animals. Treatment with losartan further increased RBF at the basal level of RAP (11.47 ± 0.88 ml/min per g) and the autoregulatory efficiency was maintained even at the lowest level of RAP (Fig. 2b). Similarly, as shown in Figure 2d, untreated I3C-induced Cyp1a1-Ren-2 transgenic rats responded to reductions in RAP by significant decreases in GFR and the treatment either with c-AUCB or with losartan markedly improved the autoregulatory efficiency of GFR in these animals.

Figure 3 shows the autoregulatory efficiency of RBF and GFR using autoregulatory indices calculated by the method of Semple and de Wardener [29]. Results of autoregulatory indices show that untreated I3C-induced Cyp1a1-Ren-2 transgenic rats exhibited substantial impairment of the autoregulatory efficiency of RBF as well as GFR already by the reduction of RAP from 120 to 100 mmHg. This was even more pronounced by the reduction of RAP to the final level of 85 mmHg. The treatment with c-AUCB did not alter autoregulatory

Fig. 2



Relationship between renal arterial pressure and renal blood flow (a and b) and glomerular filtration rate (c and d) in Cyp1a1-Ren-2 transgenic rats fed either a normal rat chow (noninduced) or a diet containing 0.3% indole-3-carbinol (I3C-induced). c-AUCB indicates treatment with the sEH inhibitor c-AUCB, losartan indicates treatment with AT₁ receptor antagonist. * $P < 0.05$ vs. basal values. # $P < 0.05$ vs. corresponding values from untreated rats. @ $P < 0.05$ vs. corresponding values from c-AUCB-treated rats.

index in noninduced animals but restored autoregulatory index of RBF in I3C-induced Cyp1a1-Ren-2 transgenic rats and normalized in these rats autoregulatory index of GFR for the reduction of RAP from 120 to 100 mmHg. However, treatment with c-AUCB did not improve the impaired autoregulatory index of GFR at the reduction of RAP from 100 to 85 mmHg in I3C-induced Cyp1a1-Ren-2 transgenic rats. In contrast, treatment with losartan not only restored autoregulatory indices of RBF and GFR in I3C-induced Cyp1a1-Ren-2 transgenic rats, but even improved autoregulatory index of RBF in noninduced animals as compared with untreated noninduced Cyp1a1-Ren-2 transgenic rats.

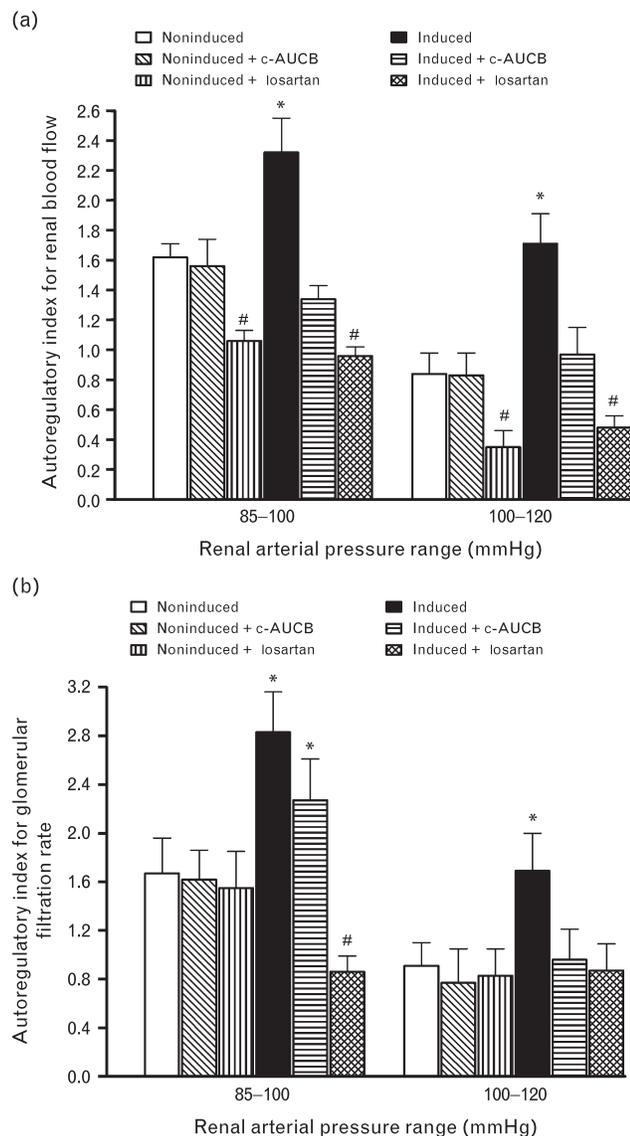
As shown in Fig. 4a, b and c, absolute sodium excretion, urine flow, and fractional sodium excretion at the spontaneous levels of RAP were not significantly different between untreated and c-AUCB-treated noninduced Cyp1a1-Ren-2 transgenic rats and the reduction in RAP elicited similar responses in both groups of animals. In contrast, treatment with losartan resulted in substan-

tial decreases in absolute sodium excretion, urine flow, and fractional sodium excretion at the spontaneous levels of RAP as compared with untreated noninduced Cyp1a1-Ren-2 transgenic rats.

As shown in Fig. 4d–f, absolute sodium excretion, urine flow, and fractional sodium excretion at basal level of RAP were not significantly different between untreated and c-AUCB and losartan-treated I3C-induced Cyp1a1-Ren-2 transgenic rats. The reduction in RAP resulted in significantly greater decreases in absolute, fractional sodium excretion and in urine flow in untreated I3C-induced Cyp1a1-Ren-2 transgenic rats than in c-AUCB-treated and losartan-treated I3C-induced Cyp1a1-Ren-2 transgenic rats.

Untreated and c-AUCB-treated or losartan-treated noninduced as well as I3C-induced Cyp1a1-Ren-2 transgenic rats exposed to the control protocol did not show any significant changes in renal hemodynamics or renal sodium excretion throughout the experiment and therefore data are not presented.

Fig. 3



Autoregulatory indexes calculated by the method of Semple and de Wardener for RBF (a) and GFR (b) responses to reductions of renal arterial pressure from 120 to 100 and from 100 to 85 mmHg. * $P < 0.05$ vs. unmarked values. # $P < 0.05$ vs. all values.

Series 2: effects of chronic soluble epoxide hydrolase inhibition and AT₁ receptor blockade on cardiac hypertrophy, proteinuria, renal glomerular and tubulointerstitial injury as well as concentrations of epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids in renal tissue

Our results show that untreated I3C-induced Cyp1a1-Ren-2 transgenic rats developed distinct cardiac hypertrophy, expressed as the ratio of left ventricle weight (LVW) to tibial length, pronounced proteinuria and marked degree of renal glomerular and tubulointerstitial injury as compared with noninduced normotensive Cyp1a1-Ren-2 transgenic rats. Treatment with *c*-AUCB

significantly attenuated the development of cardiac hypertrophy, proteinuria and the rise in glomerulosclerosis index (GSI) and tubulointerstitial injury in I3C-induced Cyp1a1-Ren-2 transgenic rats. Treatment with losartan completely prevented the development of cardiac hypertrophy, increases in proteinuria and abolished the rise in GSI and tubulointerstitial injury in I3C-induced Cyp1a1-Ren-2 transgenic rats. Noninduced normotensive Cyp1a1-Ren-2 transgenic rats exhibited minor proteinuria and minimal degree of renal glomerular and tubulointerstitial injury and either treatment with *c*-AUCB or losartan did not markedly alter these parameters (Fig. 5).

Figure 6 shows representative histological kidney slides from noninduced Cyp1a1-Ren-2 transgenic rats (a), untreated I3C-induced Cyp1a1-Ren-2 transgenic rats (b), and *c*-AUCB-treated and losartan-treated I3C-induced Cyp1a1-Ren-2 transgenic rats (c and d).

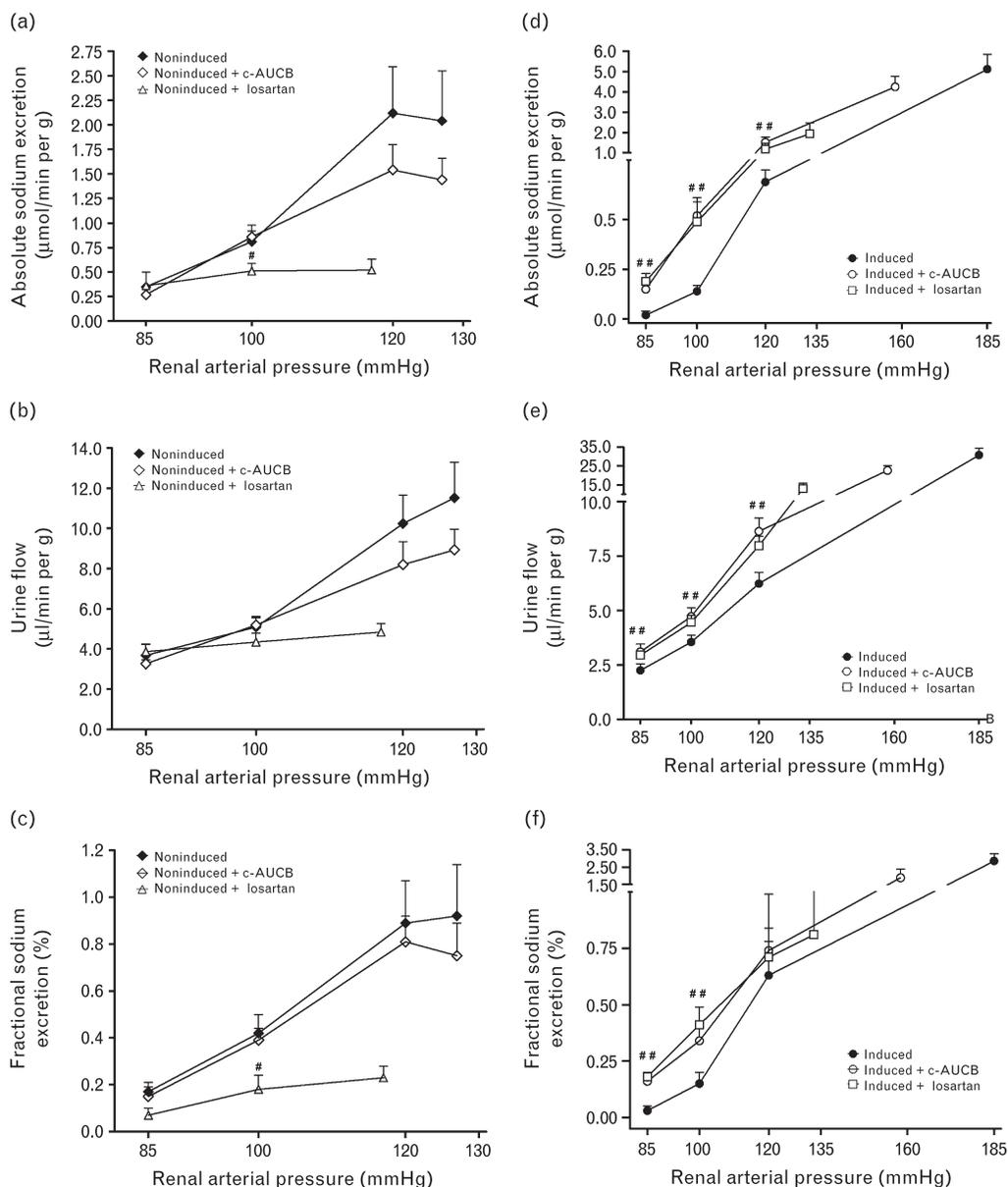
Our results show that untreated and losartan-treated I3C-induced Cyp1a1-Ren-2 transgenic rats exhibited a significant decrease in the concentration of EETs in renal cortex. Treatment with *c*-AUCB prevented the decrease in renal levels of EETs in I3C-induced Cyp1a1-Ren-2 transgenic rats. In addition, the treatment with *c*-AUCB in noninduced as well as in I3C-induced Cyp1a1-Ren-2 transgenic rats resulted in a significant decrease of the concentration of DHETEs in renal tissue. When the results are expressed as EETs/DHETEs ratio, which is the best way to describe the intrarenal availability of biologically active epoxygenase metabolites, it is shown that this ratio was markedly lower in untreated I3C-induced rats than in untreated noninduced Cyp1a1-Ren-2 transgenic rats. This ratio was not significantly altered by treatment with losartan, but the treatment with *c*-AUCB significantly increased this ratio in noninduced as well as in I3C-induced Cyp1a1-Ren-2 transgenic rats (Fig. 7).

Discussion

The first major finding of this study is that chronic treatment with the sEH inhibitor *c*-AUCB attenuated the development of malignant hypertension and prevented decreases in RBF in I3C-induced Cyp1a1-Ren-2 transgenic rats. In addition, autoregulatory indices calculated by the method of Semple and de Wardener [29] for the reductions of RAP from 120 to 100 mmHg and from 100 to 85 mmHg clearly show an impairment of the autoregulatory efficiency of RBF and GFR in untreated I3C-induced Cyp1a1-Ren-2 transgenic rats as compared with noninduced normotensive animals.

As a second major finding of the present study we observed that chronic inhibition of sEH activity by *c*-AUCB not only prevented the decreases in RBF but also allowed markedly better maintenance of autoregulation of RBF and GFR at any given level of RAP in the

Fig. 4



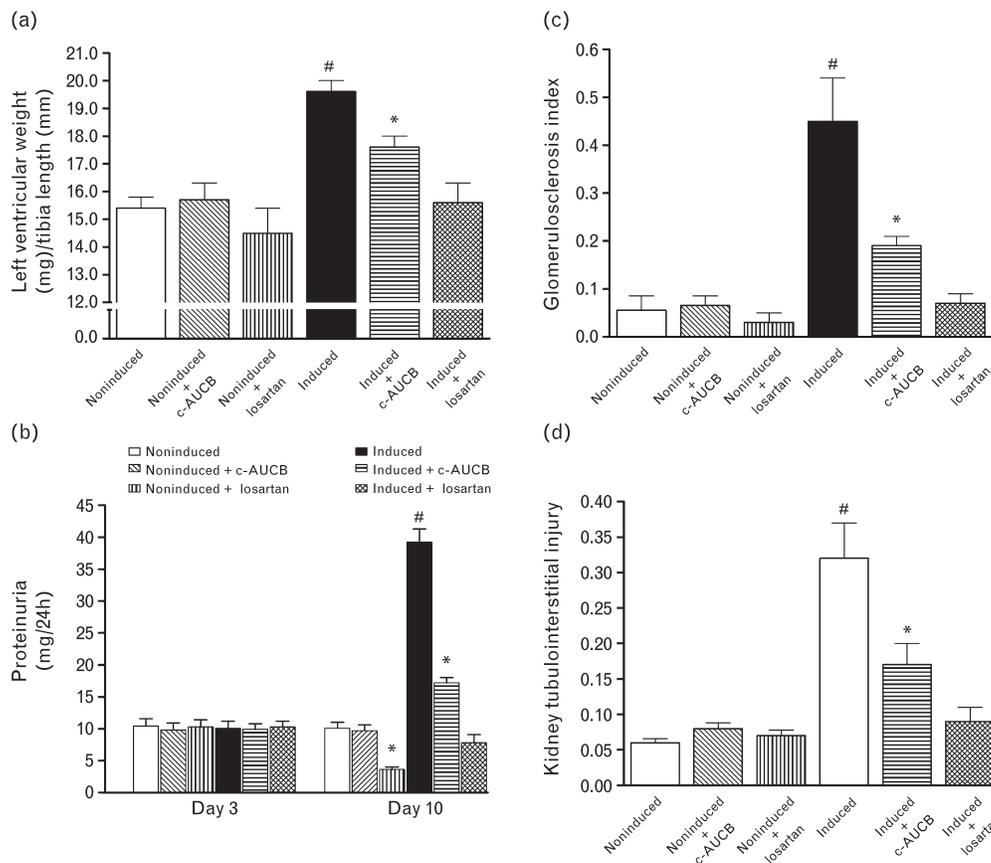
Relationship between renal arterial pressure and absolute (a and d) and fractional sodium excretion (c and f) and urine flow (b and e) in Cyp1a1-Ren-2 transgenic rats fed either a normal rat chow (noninduced) or a diet containing 0.3% indole-3-carbinol (I3C-induced). c-AUCB indicates treatment with the sEH inhibitor c-AUCB, losartan indicates treatment with AT₁ receptor antagonist. #*P* < 0.05 vs. corresponding values from untreated rats.

I3C-induced rats than in untreated I3C-induced Cyp1a1-Ren-2 transgenic rats. Moreover, we found that chronic treatment with c-AUCB significantly improved the suppressed slope of the pressure–natriuresis relationship in I3C-induced rats as compared with untreated I3C-induced Cyp1a1-Ren-2 transgenic rats. Taken together, our present findings support our original hypothesis that the antihypertensive actions of chronic inhibition of sEH activity by c-AUCB in this model of ANG II-dependent malignant form of hypertension is mediated by the improvement of the impaired autoregulatory efficiency

of RBF and GFR and by attenuation of the impairment, that is the shift to the right, of the slope of the pressure–natriuresis relationship.

In this context, it should be pointed out that an important issue of our findings is in fact related to the question as to what are the underlying mechanism(s) responsible for the improvement of the impaired autoregulatory efficiency of RBF and GFR and the suppressed pressure–natriuresis relationship in I3C-induced Cyp1a1-Ren-2 transgenic rats treated chronically by the sEH inhibitor.

Fig. 5



Heart left ventricle weight to tibia length ratio (a), proteinuria on basal conditions (day 3) and at the end of experiment (day 10) (b), glomerulosclerosis index (c) and kidney tubulointerstitial injury (d) in Cyp1a1-Ren-2 transgenic rats fed either a normal rat chow (noninduced) or a diet containing 0.3% indole-3-carbinol (I3C-induced). *c*-AUCB indicates treatment with the sEH inhibitor *c*-AUCB, losartan indicates treatment with AT₁ receptor antagonist. **P* < 0.05 vs. unmarked values. #*P* < 0.05 vs. all values.

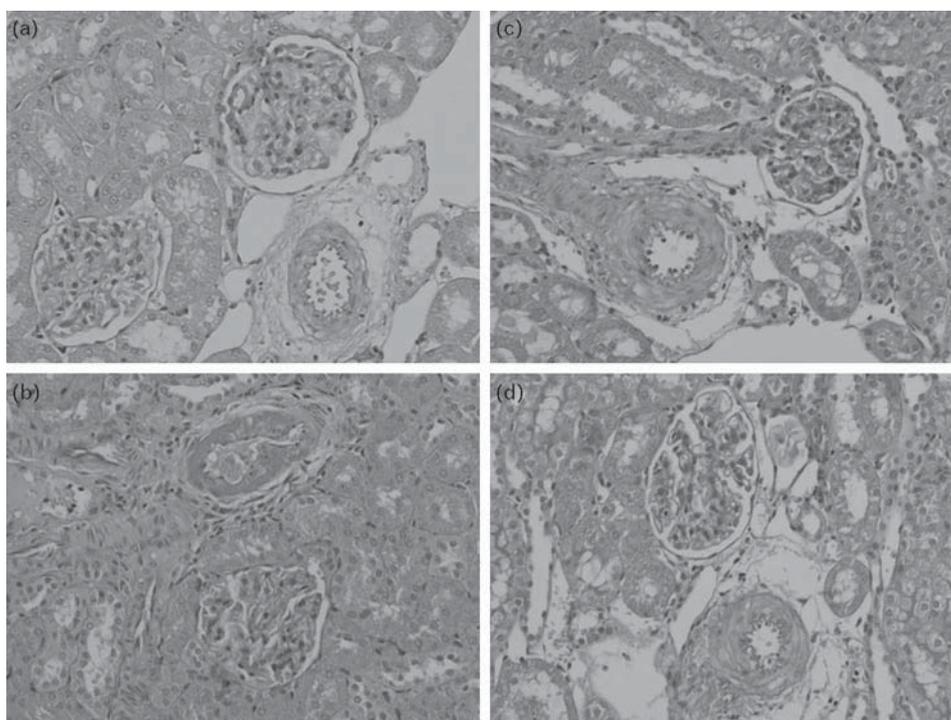
In this regard it is of critical importance to recognize that studies performed during the past 25 years have revealed that EETs are not only involved in regulating vascular tone and antagonizing the vasoconstrictor actions of ANG II, but they also directly influence tubular transport of sodium [19–23]. Our previous [17] and present results show that untreated I3C-induced Cyp1a1-Ren-2 transgenic rats exhibit a reduced intrarenal availability of biologically active epoxygenase metabolites – expressed as the ratio of EETs/DHETEs – when compared with noninduced rats. This ratio was significantly increased by the treatment with *c*-AUCB. It is highly conceivable that the increase in biological availability of EETs is responsible for the improvement of basal RBF, of the autoregulatory efficiency of RBF and GFR in response to reductions in RAP and of the slope of the pressure–natriuresis relationship in I3C-induced Cyp1a1-Ren-2 transgenic rats. This notion is also supported by our recent observation that the treatment with *c*-AUCB markedly increased EETs/DHETEs without altering the

elevated circulating and renal RAS activity in I3C-induced Cyp1a1-Ren-2 transgenic rats [17].

This suggests that changes in renal function and BP in response to *c*-AUCB should be primarily ascribed to changes in the bioavailability of EETs rather than alterations of RAS activity. There are two major underlying mechanisms how increased EETs bioavailability could improve the impaired autoregulatory efficiency of renal hemodynamics and the suppressed pressure–natriuresis relationship in I3C-induced Cyp1a1-Ren-2 transgenic rats.

First, it has been shown that at the vascular level EETs elicit vasodilatation through activation of the large-conductance calcium-activated potassium channel which is thought to attenuate calcium entry into vascular smooth muscle cells via voltage-sensitive channels [19,21]. Moreover, EETs have been implicated as the endothelium-derived hyperpolarizing factor mediating nitric oxide and prostaglandin-independent vasodilatation [21,23,30] and it has been also demonstrated that

Fig. 6



Representative histological slides of kidney from noninduced Cyp1a1-Ren-2 transgenic rats (a), untreated I3C-induced Cyp1a1-Ren-2 transgenic rats (b), *c*-AUCB- and losartan-treated I3C-induced Cyp1a1-Ren-2 transgenic rats (c and d).

EETs oppose the vasoconstrictor actions of ANG II [22,31]. On the basis of these data we suggest that the first potential mechanism is likely related to vasodilatory effects of increased EETs following *c*-AUCB treatment and normalization of RBF in I3C-induced Cyp1a1-Ren-2 transgenic rats.

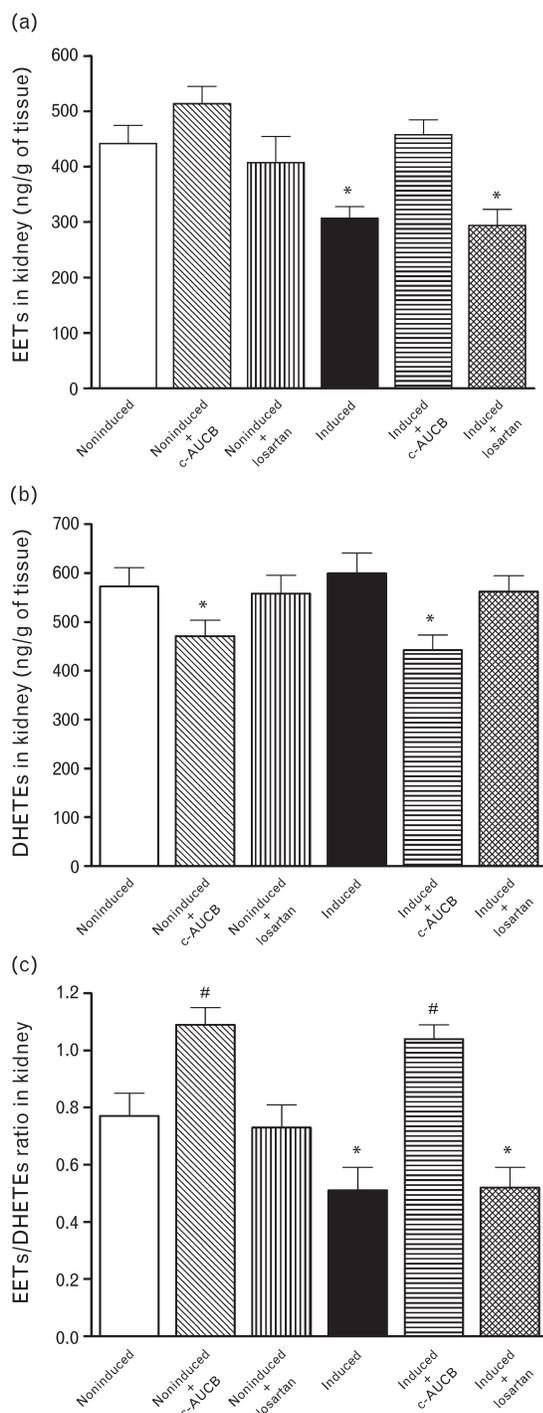
Second, previous studies have shown that at the kidney level EETs inhibit sodium reabsorption in the proximal tubule by blocking the sodium-hydrogen exchanger [32] and also decrease sodium reabsorption in the cortical collecting duct by blocking the epithelial sodium channels (ENaCs) [33]. It has been demonstrated that ENaC plays an important role in mediating the changes in sodium excretion in response to changes in RAP [6,34]. Although we are not able to determine the specific transporter involved in *c*-AUCB-induced natriureis, an improvement in tubular function was likely responsible for the improvement of the impaired pressure–natriuresis relationship in I3C-induced Cyp1a1-Ren-2 transgenic rats.

Taken together, even if the present study does not allow determination of the relative contribution of vasodilatory and tubular effects of *c*-AUCB-induced increased EET bioavailability on kidney function, it is highly conceivable that combination of both actions are underlying mechanism(s) responsible for the improvement of the

impaired autoregulatory efficiency of RBF and GFR and the suppressed pressure–natriuresis relationship in I3C-induced Cyp1a1-Ren-2 transgenic rats treated chronically by the sEH inhibitor. This notion is strongly supported by our recent findings in two-kidney, one-clip (2K1C) Goldblatt hypertensive rats – another model of ANG II-dependent hypertension, in which we found that *c*-AUCB displays antihypertensive properties that are mediated by an improvement of RBF and sodium excretion [35].

The third major finding of the present study is that chronic treatment with the AT₁ receptor antagonist losartan completely prevented the development of hypertension and the impairment of RBF and the autoregulatory efficiency of RBF and GFR and of the pressure–natriuresis relationship in I3C-induced Cyp1a1-Ren-2 rats. In addition, we have found that chronic treatment with losartan did not alter the concentrations of EETs and DHETEs in I3C-induced rats when compared with untreated I3C-induced Cyp1a1-Ren-2 transgenic rats, suggesting that the antihypertensive actions of losartan were not attributed to an increase in biological availability of intrarenal EETs. Taken together, these findings indicate that AT₁ receptor-mediated alterations in the autoregulatory efficiency of renal hemodynamics and tubular sodium reabsorption are responsible for the

Fig. 7



Epoxyeicosatrienoic acids (EETs) (a) and dihydroxyeicosatrienoic acids (DHETEs) (b) and ratio of EETs/DHETEs in kidney cortex (c) at the end of experimental period in Cyp1a1-Ren-2 transgenic rats fed either a normal rat chow (noninduced) or a diet containing 0.3% indole-3-carbinol (I3C-induced). c-AUCB indicates treatment with the sEH inhibitor c-AUCB, losartan indicates treatment with AT₁ receptor antagonist. * $P < 0.05$ vs. unmarked values. # $P < 0.05$ vs. all values.

impairment of the pressure–natriuresis relationship in I3C-induced Cyp1a1-Ren-2 transgenic rats. This notion is in good agreement with our recent findings showing that intrarenal augmentation of ANG II and ANG II-mediated derangements of the autoregulation of renal hemodynamics and the pressure–natriuresis relationship precedes the development of the malignant form of hypertension in Cyp1a1-Ren-2 transgenic rats [25].

The fourth major finding is that chronic treatment with c-AUCB not only significantly attenuated the development of hypertension, but also exhibited considerable cardioprotective and nephroprotective effects, that is, reduction of cardiac hypertrophy, of proteinuria and of renal glomerular and tubulointerstitial injury in I3C-induced Cyp1a1-Ren-2 transgenic rats. These observations are in agreement with the results of our recent studies performed in transgenic rats with constitutive expression of the mouse Ren-2 [TGR; strain name TGR(mRen2)] [27] – another commonly used transgenic model of ANG II-dependent hypertension. They have shown that altered production and/or action of CYP-derived eicosanoids contribute to the development of hypertension and of the associated end-organ damage in the monogenetic model of ANG II-dependent hypertension [27,28]. However, our present findings are of particular interest, since to our knowledge this is the first study clearly showing that chronic pharmacological inhibition of sEH, which is able to significantly enhance the concentrations of biologically active epoxygenase metabolites, exerts long-term antihypertensive action and renal and cardiovascular protection in this model of ANG II-dependent malignant hypertension. With regard to the underlying mechanism(s) responsible for the beneficial effects of c-AUCB treatment on the development of end-organ damage two potential mechanisms can be responsible. The first one can be ascribed to BP-lowering effects of chronic inhibition of sEH and previous studies have demonstrated that antihypertensive actions of sEH are always accompanied by marked reductions in end-organ damage [23,36,37] and it is in accordance with our recently published study showing that cardio and renoprotection is predominantly BP-dependent [38]. The second one is based on a recent study in the diabetic Goto-Kaizaki rats infused with ANG II and exposed to high-salt diet, in which Olearczyk *et al.* [39] have demonstrated that sEH inhibition could provide renal end-organ protection without lowering BP and it was associated with decreased renal macrophage infiltration. However, our current study does not allow determination of the specific mechanism(s) responsible for end-organ protection elicited by sEH inhibition and future studies are needed to resolve this issue.

Of special interest is also our observation that chronic treatment with losartan completely prevented the development of hypertension and of hypertension-induced end-organ in I3C-induced Cyp1a1-Ren-2 transgenic rats.

These data further strongly support our notion that inappropriate activation of AT₁ receptors by enhanced circulating and renal ANG II concentrations plays a critical role in the development of hypertension and hypertension-associated end-organ damage in Cyp1a1-Ren-2 transgenic rats with malignant hypertension.

In summary, our present findings indicate, first, that chronic treatment with the sEH inhibitor *c*-AUCB substantially attenuates the development of malignant hypertension and hypertension-induced end-organ damage in I3C-induced Cyp1a1-Ren-2 transgenic rats.

Second, treatment with the sEH inhibitor markedly improves the autoregulatory efficiency of RBF and GFR and the blunted pressure–natriuresis relationship in these transgenic rats with inducible hypertension. Our data also indicate that the improvement of the right-shifted slope of the pressure–natriuresis relationship is likely to be the essential mechanism responsible for the antihypertensive effects of chronic sEH inhibition by *c*-AUCB in this ANG II-dependent malignant form of hypertension.

In conclusion, the information derived from our present study should be considered in attempts to develop new therapeutic approaches or tools for the treatment of hypertension.

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Expanded Online Material Section

Animals and diets.

Experiments were performed in male Cyp1a1-Ren-2 transgenic rats with an initial age of 3 to 3.5 months. All animals used in the present study were bred at the Center for Experimental Medicine of the Institute for Clinical and Experimental Medicine from stock animals supplied from the Center for Cardiovascular Science, University of Edinburgh, UK (we acknowledge the generous gift of Professor Mullins). All diets used in the present study were produced by SEMED (Prague 4, Czech Republic). Rats were fed either a rat chow without I3C (noninduced groups) or a rat chow containing 0.3 % I3C (induced groups). It has been shown that I3C is a dietary supplement, which in transgene-negative rats does not exhibit any harmful biological effects but causes a very strong induction of Cyp1a1 through activation of the aryl hydrocarbon receptor, which is a basic helix-loop-helix-transcription factor that binds to the Cyp1a1 promoter [1]. Previous studies have demonstrated that continuous administration of this dose of I3C in the diet resulted in a marked expression of the Ren-2 renin gene and the development of an ANG II-dependent malignant form of hypertension in transgene-positive animals, whereas the transgene-negative rats (Fisher F344 rats) remained normotensive [2–6].

Series 1: Surgical preparation and experimental design for acute autoregulatory experiments.

Rats were anesthetized with thiopental sodium (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 and the exterior end of the tracheal cannula was placed inside a small plastic chamber into which a humidified 95% oxygen/5% carbon dioxide mixture was continuously passed, which has been shown to improve the stability of arterial BP of pentobarbital sodium-anesthetized rats [7]. The right jugular vein was catheterized for fluid infusion and anesthetic 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 was monitored with a pressure transducer (model MLT 1050) and recorded on the computer using a computerized data-acquisition system (PowerLab/4SP, AD Instruments, UK). The left kidney was exposed via a flank incision, isolated from the surrounding tissue and placed in a lucite cup, and the ureter was cannulated with a PE-10 catheter. The 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 GmbH, Germany) connected to a Transonic flowmeter was placed around the left renal artery and RBF was recorded using a computerized acquisition system. At the end of the experiment, zero value was established by complete occlusion of aorta. During surgery, an isotonic saline solution containing bovine serum albumin (6 %, Sigma Chemical Co., Prague, Czech Republic) was infused at a rate of 40 µl/min. After the 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 the animals to establish steady state before initiating one 30-min control urine collection at physiological level of RAP. In groups in whom the control protocol was applied, three additional 30-min urine collection periods at physiological level of RAP were performed. In groups in whom the experimental protocol was applied, a first 30-min urine collection at the physiological level of RAP was performed. Thereafter, 30-min urine collections were performed at reduced RAPs of 120, 100 and 85 mmHg. Five minutes of equilibration time were allowed after each step of reduction in RAP. Blood samples were collected after the second and fourth urine collection to allow determination of GFR and renal sodium excretion. This experimental procedure is identical to that used by Wang et al. [8] in ANG II-infused hypertensive rats and that recently employed in our lab in Cyp1a1-Ren-2 transgenic rats [9].

Urine volume was measured gravimetrically. Urinary sodium concentration was 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 was calculated using standard formula. The autoregulatory index (AI) of RBF was calculated by the method of Semple and de Wardener [10] using the following formula: $AI = [(RBF_2 - RBF_1)/RBF_1]/[(RAP_2 - RAP_1)/RAP_1]$. The same formula was employed for calculation of the AI of GFR. An AI of zero indicates ideal autoregulation and higher values indicate impairment of the autoregulation.

Series 2: Evaluation of renal glomerular damage and tubulointerstitial injury.

The kidney was quickly removed, fixed in 4% formaldehyde, dehydrated and embedded in paraffin. The sections stained with hematoxylin – eosin and PAS (periodic acid – Schiff reaction) were examined and evaluated in a blind-test fashion. Fifty glomeruli in each kidney were examined on a semi-quantitative scale as described previously [11,12]:

grade 0, all glomeruli normal; *grade 1*, sclerotic area up to 25% (minimal sclerosis); *grade 2*, sclerotic area 25 to 50% (moderate sclerosis); *grade 3*, sclerotic area 50 to 75% (moderate-to-severe sclerosis); *grade 4*, sclerotic area 75 to 100% (severe sclerosis). The glomerulosclerosis index (GSI) was calculated using the following formula: $GSI = [(1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)] / (n_0 + n_1 + n_2 + n_3 + n_4)$, where n_x is the number of glomeruli in each grade of glomerulosclerosis.

Renal cortical and tubulointerstitial injury was evaluated as defined by Nakano et al. [13] and as used in our recent study [14] for inflammatory cell infiltration, tubular dilatation and/or atrophy, or interstitial fibrosis, and was graded semi-quantitatively using the following scale of lesions: grade 0, no abnormal findings; 1, mild (<25 % of cortex); 2, moderate (25 – 50 % of cortex); 3, severe (>50 % of cortex). The lesions were assessed in at least 30 random and non-overlapping fields in the renal cortex.

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