Different mechanisms of acute versus long-term antihypertensive effects of soluble epoxide hydrolase inhibition: Studies in Cyp1a1-Ren-2 transgenic rats

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SUMMARY

Recent studies have shown that the long-term antihypertensive action of soluble epoxide hydrolase inhibition (sEH) in angiotensin-II (AngII)-dependent hypertension might be mediated by the suppression of intrarenal AngII levels. To test this hypothesis, we examined the effects of acute (2 days) and chronic (14 days) sEH inhibition on blood pressure (BP) in transgenic rats with inducible AngII-dependent hypertension. AngII-dependent malignant hypertension was induced by 10 days’ dietary administration of indole-3-carbinol (I3C), a natural xenobiotic that activates the mouse renin gene in Cyp1a1-Ren-2 transgenic rats. BP was monitored by radiotelemetry. Acute and chronic sEH inhibition was achieved using cis-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy) benzoic acid, given at doses of 0.3, 3, 13, 26, 60 and 130 mg/L in drinking water. At the end of experiments, renal concentrations of epoxyeicosatrienoic acids, their inactive metabolites dihydroxyeicosatrienoic acids and AngII were measured. Acute BP-lowering effects of sEH inhibition in I3C-induced rats was associated with a marked increase in renal epoxyeicosatrienoic acids to dihydroxyeicosatrienoic acids ratio and acute natriuresis. Chronic treatment with cis-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy) benzoic acid in I3C-induced rats elicited dose-dependent persistent BP lowering associated with a significant reduction of plasma and kidney AngII levels. Our findings show that the acute BP-lowering effect of sEH inhibition in I3C-induced Cyp1a1-Ren-2 transgenic rats is mediated by a substantial increase in intrarenal epoxyeicosatrienoic acids and their natriuretic action without altering intrarenal renin–angiotensin system activity. Long-term antihypertensive action of cis-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy) benzoic acid in I3C-induced Cyp1a1-Ren-2 transgenic rats is mediated mostly by suppression of intrarenal AngII concentration.

Key words: angiotensin-II, cytochrome P-450 epoxygenase, eicosanoids, epoxyeicosatrienoic acids, hypertension, soluble epoxide hydrolase.

INTRODUCTION

An increasing body of evidence indicates that cytochrome P-450 (CYP)-dependent metabolites of arachidonic acid, including epoxyeicosatrienoic acids (EET), play an important role in the regulation of cardiovascular and renal functions.1–4 Numerous independent studies have shown that increasing bioavailability of EET in tissues by preventing their degradation to the biologically inactive dihydroxyeicosatrienoic acids (DHETE) using soluble epoxide hydrolase (sEH) inhibition has antihypertensive effects.5–15 Based on these studies, it has been proposed that the EET-mediated antihypertensive actions are related to their immediate vasodilatory effects in addition to their direct influence on renal tubular transport of sodium.3,4,16

We recently found that the sEH inhibitor cis-4-(4-(3-adamantan-1-yl-ureido)cyclohexyloxy) benzoic acid (c-AUCB), induced a significant reduction in plasma angiotensin-II (AngII) levels and normalization of kidney AngII concentration in a Ren-2 transgenic rat model of angiotensin-II (AngII)-dependent hypertension.16

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Received 24 July 2014; revision 26 August 2014; accepted 29 August 2014.

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hypertension (TGR).\textsuperscript{17} Though c-AUCB significantly increased the intrarenal availability of biologically active EET in TGR, detailed analyses of the results of that study made us propose that the antihypertensive action of sEH inhibition in TGR is predominantly mediated by suppression of the systemic and intrarenal renin–angiotensin system (RAS).\textsuperscript{17} As the dose of c-AUCB used in that study was in the upper range of the dosage recommended (26 mg/L in drinking water),\textsuperscript{18} double the dose we usually utilize,\textsuperscript{9,12,14,15} we considered it important to examine whether the effect of sEH inhibition on the RAS might be dose-dependent.

Of note, the most common model of AngII-dependent hypertension is the one obtained by chronic infusion of subpressor doses of AngII that lead to suppression of endogenous RAS.\textsuperscript{19–21} Another possibility is the two-kidney, one-clip (2K1C) Goldblatt hypertension, where blood pressure (BP) elevation is the consequence of surgically-induced unilateral renal artery stenosis.\textsuperscript{22} Thus, induction of AngII-dependent hypertension in these models results either from application of exogenous AngII leading to unphysiological suppression of RAS or from a surgical intervention that yields a variable degree of RAS activation.\textsuperscript{19–24} Furthermore, the exact onset and the severity of hypertension in these models often vary and cannot be precisely controlled. To avoid these limitations, we used an inbred transgenic rat line (strain name: TGR(Cyp1a1-Ren-2)) that offers a unique possibility to precisely control the development of AngII-dependent hypertension.\textsuperscript{25} This transgenic rat model allows assessment of the pathophysiological mechanisms of AngII-dependent hypertension involving increased activity of endogenous RAS. Furthermore, we and others have shown that the gene expression, the level of endogenously produced AngII and consequently the degree of hypertension can be precisely controlled.\textsuperscript{11,12,15,26–30}

Our aim was to discover if sEH inhibition would result in dose-dependent inhibition of the circulating and intrarenal RAS in hypertensive Cyp1a1-Ren-2 transgenic rats. Therefore, we evaluated the effects of acute (2 days) and chronic (14 days) c-AUCB treatment on BP, and AngII, EET and DHETE concentrations as affected by doses ranging from 0.3 to the highest suggested dose of 130 mg/L in drinking water.\textsuperscript{18} Furthermore, to elucidate the mechanism(s) responsible for the effects of sEH inhibition on RAS activity, Ren-2 renin gene expression and activities/concentrations of individual components of the RAS were assessed in untreated and c-AUCB-treated Cyp1a1-Ren-2 transgenic rats; this was carried out under control conditions and after induction of the renin gene. Finally, our recent data\textsuperscript{7} suggested that after high-dose c-AUCB treatment, the pattern of changes was similar as that observed in AngII-dependent hypertension treated with angiotensin-converting enzyme inhibitor (ACEi).\textsuperscript{19,20,31,32} Therefore, we also determined the effects of ACEi treatment in non-induced animals and in Cyp1a1-Ren-2 transgenic rats after induction of the renin gene on the profiles of hypertension and EET and DHETE concentrations, and expression and activities/concentrations of individual components of the RAS. This was carried out to gain a deeper insight into the possible role of the interaction between CYP-dependent metabolites and the RAS in the antihypertensive potency of sEH inhibition.

![Fig. 1](https://example.com/figure1.png)

**RESULTS**

**Series 1: Effects of c-AUCB and ACEi in non-induced or I3C-induced Cyp1a1-Ren-2 transgenic rats on arterial blood pressure, body weight, urinary sodium excretion, plasma c-AUCB concentrations and Ren-2 renin gene expression**

As shown in Fig. 1, basal values of mean arterial pressure (MAP) were not significantly different among experimental groups. In untreated animals, I3C-induction resulted in the rise of MAP from 102 ± 3 mmHg (day −10) to 171 ± 3 mmHg.
(day 0; \( P < 0.05 \)). Non-induced rats remained normotensive throughout the experiment and the treatment with the highest dose of \( c\text{-AUCB} \) (130 mg/L) did not have any significant effect on their MAP. However, the treatment with ACEi (trandolapril, 6 mg/L) resulted in a significant decrease in MAP as compared to untreated non-induced rats (78 ± 3 vs 105 ± 3 mmHg, on day 14; Fig. 1a). As also shown in Fig. 1a, treatment with 0.3 mg/L of \( c\text{-AUCB} \) did not affect the course of hypertension in I3C-induced rats as compared to untreated I3C-induced rats (175 ± 4 vs 177 ± 3 mmHg measured on day 14). Treatment with 3 mg/L of \( c\text{-AUCB} \) significantly decreased MAP in I3C-induced rats, with maximal BP-lowering effect observed 2 days after initiation of \( c\text{-AUCB} \) administration (from 173 ± 4 to 137 ± 2 mmHg, \( P < 0.05 \)). On day 7, MAP returned to levels observed in I3C-induced untreated rats. Treatment with 13 mg/L of \( c\text{-AUCB} \) caused marked decreases in MAP with a maximum BP-lowering effect on day 4 of treatment (from 174 ± 4 to 114 ± 2 mmHg, \( P < 0.05 \)), followed by a gradual return to levels observed in untreated I3C-induced rats (Fig. 1a).

As shown in Fig. 1b, treatment with 26 mg/L \( c\text{-AUCB} \) elicited a profound decrease in MAP to normotensive levels (from 173 ± 4 to 113 ± 3 mmHg, \( P < 0.05 \)), then MAP slowly increased, but at the end of experiments still remained significantly lower compared to untreated I3C-induced rats (148 ± 3 vs 177 ± 3 mmHg, \( P < 0.05 \)). Treatment with 60 mg/L of \( c\text{-AUCB} \) had similar rapid BP-lowering effects; however, the MAP remained within the normotensive range until the end of the experiment (118 ± 3 mmHg on day 14). Treatment with 130 mg/L \( c\text{-AUCB} \) and administration of ACEi caused similar profound decrease in MAP to normotensive levels, and at the end of the experiment it even dropped below those observed in untreated non-induced rats (91 ± 3 and 89 ± 3 vs 105 ± 3 mmHg, \( P < 0.05 \) in both cases; Fig. 1b).

As shown in Supplemental Fig. 1a, untreated and \( c\text{-AUCB} \)-and ACEi-treated non-induced rats showed a significant body-weight (BW) gain throughout the experiment, with final increases by +57 ± 3, +56 ± 3 and +54 ± 4 g, respectively (\( P < 0.05 \) vs initial BW). In I3C-induced rats, the development of hypertension was associated with a profound loss of BW (from 320 ± 8 to 207 ± 7 g, \( P < 0.05 \)). In I3C-induced rats, neither the treatment with the highest dose of \( c\text{-AUCB} \) (130 mg/L) nor that with ACEi fully prevented the BW losses (Fig. S1b).

As shown in Fig. 2, the basal daily sodium excretion did not significantly differ among the groups, and remained unaltered in untreated non-induced as well as I3C-induced rats. The treatment with 130 mg/L of \( c\text{-AUCB} \) in non-induced rats as well as the treatment with the lowest dose of \( c\text{-AUCB} \) (0.3 mg/L) in I3C-induced rats did not significantly alter daily sodium excretion (Fig. 2a). In contrast, the treatment with each dose of \( c\text{-AUCB} \) (from 3 mg/L to 130 mg/L) and ACEi elicited marked increase in daily sodium excretion in I3C-induced rats when measured on the second day of treatment (Fig. 2a, b). In addition, the treatment with ACEi caused a substantial increase in daily sodium excretion in non-induced rats on the second day of treatment (Fig. 2a). However, already on day 8, sodium excretion returned to the levels observed in untreated non-induced rats, and remained at those levels until the end of the experiment.

As shown in Fig. 3a, plasma concentrations of \( c\text{-AUCB} \) in groups that were not treated with this sEH inhibitor were at zero level, whereas in \( c\text{-AUCB} \)-treated groups they increased in a dose-dependent manner. Starting with the dose 3 mg/L, \( c\text{-AUCB} \) plasma concentration was already above the value of IC\textsubscript{50} for the specific sEH inhibition on the second day of treatment. This concentration was observed to effectively inhibit sEH activity in vitro as well as in vivo.\textsuperscript{18} Of special interest is the finding that treatment with the highest dose of \( c\text{-AUCB} \) (130 mg/L) resulted in maximum plasma concentrations on day 7 of administration, and the levels were approximately 20-fold higher than the IC\textsubscript{50} value required for the specific sEH inhibition.\textsuperscript{18}
Series 2: Effects of c-AUCB and ACEi in non-induced or I3C-induced Cyp1a1-Ren-2 transgenic rats on EET, DHETE, plasma and kidney tissue AngII concentrations, plasma renin activity, and plasma and kidney tissue ACE activity

Figures 4a and 5a summarize the availability of biologically active epoxyenase products in kidney tissue expressed as the ratio of EET-to-DHETE. The ratios in untreated I3C-induced rats were significantly lower than in untreated non-induced rats. The treatment with 130 mg/L of c-AUCB did not significantly change the ratios in non-induced rats. As shown in Figs 4a and 5a, acute (2 days) as well as chronic (14 days) treatment with c-AUCB beginning with the dose of 3 mg/L caused significant increase in the EET-to-DHETE ratio. In addition, the increase in EET-to-DHETE ratios were similar in I3C-induced rats treated with each of the other doses of c-AUCB (from 13 to 130 mg/L). However, the increases in the EET-to-DHETE ratio were greater in acutely- than in chronically-treated I3C-induced rats for all doses of c-AUCB. The treatment with ACEi did not significantly change the ratios of EET-to-DHETE in I3C-induced rats (Figs 4a,5a).

As shown in Figs 4b and 5b, plasma AngII levels were significantly higher in untreated I3C-induced rats than in untreated non-induced rats. Neither acute (2 days) nor chronic (14 days) treatment with 130 mg/L of c-AUCB significantly changed plasma AngII levels in non-induced rats. Figure 4b shows that acute treatment with 0.3, 3 or 13 mg/L of c-AUCB did not alter plasma AngII levels in I3C-induced as compared to untreated I3C-induced rats. In addition, acute administration of 26, 60 and 130 mg/L of c-AUCB in I3C-induced rats resulted in significant decrease in plasma AngII levels as compared to untreated I3C-induced rats (106 ± 6, 77 ± 9 and 74 ± 8 vs 148 ± 9 fmol/mL, different at P < 0.05 in each case), but they remained significantly higher than observed in untreated non-induced rats. Acute treatment with ACEi normalized plasma AngII levels to values observed in untreated non-induced rats (31 ± 3 vs 24 ± 5 fmol/mL). Acute treatment with ACEi did not alter plasma AngII levels in non-induced rats (Fig. 4b).

Figure 5b shows that similarly to acute treatment, chronic administration of 0.3, 3 and 13 mg/L of c-AUCB did not significantly change plasma AngII levels in I3C-induced rats. In contrast to acute treatment, chronic administration of 26, 60 and 130 mg/L of c-AUCB decreased plasma AngII levels to values observed in untreated non-induced rats. As with acute treatment, chronic treatment with ACEi in I3C-induced rats also decreased plasma AngII levels to values observed in untreated non-induced rats. Plasma AngII levels in I3C-induced rats decapitated on day 2 were higher compared to the values on day 14; in untreated I3C-induced rats, the respective values were 148 ± 9 and 87 ± 8 fmol/mL (different at P < 0.05). Chronic treatment with ACEi did not significantly change plasma AngII levels in non-induced rats (Fig. 5b).

As shown in Figs 4c and 5c, kidney AngII concentrations were substantially higher in untreated I3C-induced than in untreated non-induced rats. Acute treatment with any dose of c-AUCB did not affect kidney AngII concentrations in I3C-induced rats. In contrast, acute treatment with ACEi significantly decreased kidney AngII concentrations as compared to untreated I3C-induced rats (197 ± 19 vs 514 ± 34 fmol/g of tissue, P < 0.05), but they still remained significantly higher compared to values observed in

As shown in Fig. 3b, the liver expression of Ren-2 renin gene in the non-induced rats was virtually undetectable. Treatment with 130 mg/L of c-AUCB and ACEi did not change liver Ren-2 renin gene expression in non-induced rats. I3C administration resulted in marked liver Ren-2 renin gene expression, and it was substantially potentiated by effective sEH and ACE inhibition.
Fig. 4 Effects of acute (2 days) treatment with cis-4-(4-(3-adamantan-1-yl-ureido) cyclohexyloxy) benzoic acid (c-AUCB) or angiotensin-converting enzyme inhibitor (ACEi) on (a) kidney tissue epoxyeicosatrienoic acids (EET)-to-dihydroxyeicosatrienoic acids (DHETE) ratio, (b) plasma angiotensin-II (AngII) levels and (c) kidney AngII levels in indole-3-carbinol (I3C)-induced and non-induced Cyp1a1-Ren-2 transgenic rats. *P < 0.05 versus unmarked values, #P < 0.05 versus all the other values. ( ), Non-induced untreated; ( ), non-induced + c-AUCB (130 mL); ( ), non-induced + ACEi; ( ), 13C-induced untreated; ( ), 13C-induced + c-AUCB (0.3 mg/L); ( ), 13C-induced + c-AUCB (3 mg/L); ( ), 13C-induced + c-AUCB (13 mg/L); ( ), 13C-induced + c-AUCB (26 mg/L); ( ), 13C-induced + c-AUCB (60 mg/L); ( ), 13C-induced + c-AUCB (130 mg/L); ( ), 13C-induced + ACEi.

Fig. 5 Effects of chronic (14 days) treatment with cis-4-(4-(3-adamantan-1-yl-ureido) cyclohexyloxy) benzoic acid (c-AUCB) or angiotensin-converting enzyme inhibitor (ACEi) on (a) kidney tissue epoxyeicosatrienoic acids (EET)-to-dihydroxyeicosatrienoic acids (DHETE) ratio, (b) plasma angiotensin-II (AngII) levels and (c) kidney AngII levels in indole-3-carbinol (I3C)-induced and non-induced Cyp1a1-Ren-2 transgenic rats. *P < 0.05 versus unmarked values, #P < 0.05 versus all the other values. ( ), Non-induced untreated; ( ), non-induced + c-AUCB (130 mL); ( ), non-induced + ACEi; ( ), 13C-induced untreated; ( ), 13C-induced + c-AUCB (0.3 mg/L); ( ), 13C-induced + c-AUCB (3 mg/L); ( ), 13C-induced + c-AUCB (13 mg/L); ( ), 13C-induced + c-AUCB (26 mg/L); ( ), 13C-induced + c-AUCB (60 mg/L); ( ), 13C-induced + c-AUCB (130 mg/L); ( ), 13C-induced + ACEi.
non-induced rats (197 ± 19 vs 74 ± 7 fmol/g of tissue, P < 0.05). Acute treatment with ACEi did not alter kidney AngII concentrations in non-induced rats (Fig. 4c).

In contrast to the results of acute treatment, chronic treatment with 26 mg/L c-AUCB (Fig. 5c) markedly decreased kidney AngII concentrations in I3C-induced rats compared to untreated I3C-induced rats (117 ± 9 vs 443 ± 32 fmol/g of tissue, P < 0.05). Chronic administration of 60 mg/L of c-AUCB in I3C-induced rats decreased kidney AngII concentrations to values observed in non-induced rats. Furthermore, treatment with 130 mg/L c-AUCB and ACEi elicited significant decrease in kidney AngII concentrations even below the values observed in untreated non-induced rats (39 ± 4 and 37 ± 4 vs 80 ± 4 fmol/g of tissue, P < 0.05 in each case). Finally, chronic treatment with ACEi resulted in a significant decrease in kidney AngII concentrations in non-induced rats (Fig. 5c).

As shown in Figs 6a and 7a, plasma renin activities were markedly higher in untreated I3C-induced rats than in untreated non-induced rats. As expected, plasma renin activity in untreated I3C-induced rats from the chronic treatment protocol (i.e. 14 days) was noticeably higher as compared to untreated I3C-induced rats from the acute treatment protocol (2 days). Neither acute nor chronic treatment with c-AUCB significantly changed plasma renin activities in non-induced or I3C-induced rats. In contrast, acute as well as chronic treatment with ACEi elicited significant increase in plasma renin activities in non-induced rats. Remarkably, chronic treatment with ACEi increased plasma renin activity in non-induced rats to values that were observed in untreated I3C-induced rats (Fig. 7a).

As shown in Figs 6b,c and 7b, there were no significant differences in plasma and kidney ACE activities estimated as the ratio of AngII-to-AngI among non-induced and I3C-induced rats, and acute treatment with any dose of c-AUCB did not significantly alter them (Fig. 6b,c). In contrast, acute treatment with ACEi caused significant decrease in plasma and kidney ACE activities in non-induced as well as in I3C-induced rats (Fig. 6b,c).

Figure 7b and c show that chronic treatment with 0.3, 3 and 13 mg/L of c-AUCB did not change plasma and kidney ACE activities in I3C-induced rats. In contrast, chronic treatment with 26, 60 and 130 mg/L of c-AUCB profoundly decreased plasma and kidney ACE activities in I3C-induced rats, bringing them close to the levels observed in I3C-induced rats exposed to chronic ACEi treatment. Similar results regarding plasma and kidney ACE activities were obtained by direct measurement of ACE activity.

**DISCUSSION**

The first major finding of the present study was that the acute (2 days’ treatment) BP-lowering effect of sEH inhibition in our model of AngII-dependent hypertension was associated with a substantial increase in renal bioavailability of EET (increased EET-to-DHETE) and natriuresis, whereas intrarenal AngII concentration remained unaltered. This is in accordance with the vast evidence indicating antihypertensive properties of EET are largely related to their natriuretic potency. Indeed, EET have been shown to inhibit sodium reabsorption in the proximal tubule by blocking the sodium-hydrogen exchanger and in the cortical collecting duct by blocking the epithelial sodium channels. In agreement with our previous studies, the I3C-induced Cyp1a1-Ren-2 transgenic rats showed reduced availability of biologically active epoxyenase metabolites. The data accord also
with our recent finding that chronic inhibition of sEH normalized intrarenal EET bioavailability and improved the pressure-natriuresis relationship in 2K1C Goldblatt hypertensive and I3C-induced Cyp1a1-Ren-2 transgenic rats. This further supports the concept that net intrarenal deficiency of EET contributes to the impairment of the pressure-natriuresis mechanism. In accordance with the concept first proposed by Guyton et al. and validated by several other groups, the impairment of this mechanism is the crucial factor responsible for the development and maintenance of hypertension.

Acute administration of c-AUCB at the dose of 3 mg/L significantly increased intrarenal availability of biologically active EET and other epoxy fatty acids; starting at the dose 13 mg/L and higher, even the intrarenal EET in I3C-induced Cyp1a1-Ren-2 transgenic rats increased to values significantly higher than those in non-induced rats. Of note, acute administration of c-AUCB at doses below 13 mg/L did not alter plasma AngII levels in I3C-induced Cyp1a1-Ren-2 transgenic rats. However, starting at the dose 26 mg/L, these levels were reduced but still remained distinctly higher than in non-induced rats. Furthermore, acute administration of c-AUCB at any dose did not significantly change kidney AngII in our hypertensive rats. Taken together, these findings strongly suggest that acute sEH inhibition does not alter intrarenal RAS activity, whereas acute ACEi treatment results in plasma AngII normalization and in striking suppression of kidney AngII concentrations. Therefore, based on our earlier and present findings, we propose that the mechanism underlying BP-lowering in response to acute sEH inhibition is related to markedly enhanced intrarenal availability of EET and their direct inhibitory influence on renal tubular sodium transport, and is not crucially dependent on the alterations in RAS activity.

The second critically important finding of the present study was that sustained antihypertensive action of chronic (14 days’ treatment) sEH inhibition with c-AUCB is dose-dependent; it is associated with both normalization of intrarenal EET, and significant reduction of plasma and kidney AngII levels. This is in agreement with our recent findings where a high dose of c-AUCB (26 mg/L) led to a significant reduction in plasma AngII and normalization of kidney AngII concentrations in TGR, another rat model of AngII-dependent hypertension, whereas low-dose treatment (3 mg/L), which still efficiently blocked sEH, did not alter circulating and tissue RAS, the c-AUCB induced BP lowering persisted throughout the 2-week period of treatment. Two features of this response were very remarkable. First, the BP lowering effect of c-AUCB was dose-dependent. Second, the degree of concurrent suppression of AngII concentrations was also dose-dependent and parallel to the BP change. In contrast, in I3C-induced Cyp1a1-Ren-2 transgenic rats, chronic c-AUCB treatment did not alter intrarenal EET in a dose-dependent manner: the maximal effect was already observed at the dose of 13 mg/L. Admittedly, our assays of plasma c-AUCB concentration showed that each dose was highly above the range of IC50 for the specific sEH inhibition. Furthermore, although chronic c-AUCB treatment increased intrarenal EET in our hypertensive rats to values observed in non-induced rats, the effect of chronic treatment on the ratio of EET-to-DHETE was significantly smaller compared to that of acute c-AUCB treatment. Taken together, these data strongly suggest that sustained antihypertensive actions of sEH inhibition with c-AUCB were specifically mediated by the suppression of circulating and especially intrarenal AngII levels rather than by normalization of the intrarenal availability of biologically active EET. This conclusion fits...
well with recent data showing the crucial importance of an enhanced intrarenal AngII in the pathophysiology of AngII-dependent hypertension.20,27,31,32

What are the mechanisms responsible for the effects of chronic sEH inhibition by c-AUCB on plasma and kidney AngII levels in I3C-induced Cyp1a1-Ren-2 transgenic rats?

We found that chronic c-AUCB treatment caused dose-dependent suppression of plasma as well as kidney AngII concentrations. It will be recalled that inhibition of RAS activity by pharmacological blockade of AngII type 1 receptor leads to a marked elevation of circulating AngII levels. This is the consequence of the interruption of the short-loop negative feedback wherein AngII type 1 receptor activation suppresses renin secretion and decreases plasma AngII levels.46-47 It is therefore expected that the blockade of the RAS activity by chronic c-AUCB treatment must be either at the renin or at the ACE level. In this context, a further important finding of the present study is that chronic sEH inhibition by c-AUCB did not suppress the expression of the Ren-2 renin gene in I3C-induced Cyp1a1-Ren-2 transgenic rats. In contrast, these animals showed a marked increase in the expression of Ren-2 renin gene, suggesting that I3C-induced Cyp1a1-Ren-2 transgenic rats responded to suppression of AngII concentrations by a compensatory rise in renin gene expression. This is consistent with the concept of disruption of the short-loop negative feedback of AngII on the renin gene expression and secretion.46,47 In addition, our present results clearly show that chronic sEH inhibition by c-AUCB did enhance plasma renin activity in I3C-induced Cyp1a1-Ren-2 transgenic rats. These findings strongly suggest that the blockade of the RAS activity by chronic c-AUCB treatment did not occur at the renin level.

In this regard, of special interest is our finding that I3C-induced Cyp1a1-Ren-2 transgenic rats treated chronically with ACEi or with high doses of c-AUCB (either treatment leading to sustained normalization of BP) affected AngII concentrations, and plasma and kidney ACE activity in the same way. This strongly suggests that c-AUCB, a new orally active sEH inhibitor, blocks the activity of the RAS at the ACE level, an unexpected finding in a study that was not designed to explore the ACEi-like action of c-AUCB. In addition, a limitation of our present study was that it was not designed to delineate the strict correlation between decreases in BP and BW in response to dose-dependent treatment and changes in intrarenal AngII concentrations in I3C-induced Cyp1a1-Ren-2 transgenic rats. It is clear that additional studies are required to address this issue more precisely.

Furthermore, the evidence that chronic treatment with ACEi and the highest dose of c-AUCB (130 mg/L) decreased kidney AngII concentrations in I3C-induced Cyp1a1-Ren-2 transgenic rats even below the levels observed in non-induced rats further emphasizes the critical role of the intrarenal AngII content in the pathophysiology of hypertension in this model. The present results corroborate those of the previous studies showing that suppression of intrarenal RAS activity is the crucial mechanism underlying effective long-term antihypertensive treatment in AngII-dependent models of hypertension.20,22,31,32

With regard to BW loss in I3C-induced Cyp1a1-Ren-2 transgenic rats, previous studies have shown that malignant hypertension in this model is characterized by augmented activity of the RAS, rapid increase in BP and an intensive pressure diuresis associated with profound loss of BW, identical to that of our present study.25-29 In our present study, the decrease in BW in untreated I3C-induced Cyp1a1-Ren-2 transgenic rats was not simply the result of reduced food intake (and consequently loss in muscle weight etc.), because our data from monitoring food intake show that these animals reduced food intake by approximately 7%, which was not significantly different from food intake of either non-induced rats or I3C-induced and pharmacologically treated rats. Taken together, our present data show that the loss in BW is a consequence of the accentuated pressure diuresis accompanying the development of malignant hypertension. However, it is obvious that future studies should more complexly address the mechanism(s) responsible for the BW loss after induction of the renin gene in Cyp1a1-Ren-2 transgenic rats.

In conclusion, the present findings show that the acute BP-lowering effects of sEH inhibition in I3C-induced Cyp1a1-Ren-2 transgenic rats are mediated by a substantial increase of intrarenal biologically active epoxygenase products and their natriuretic action. In contrast, the long-term antihypertensive action of sEH inhibition in this rat model is chiefly due to the suppression of intrarenal AngII concentration that is mediated by the ACEi-like action of c-AUCB.

METHODS

The studies were carried out in accordance with guidelines and practices established by the Committee for Animal Care and Use at the Institute of Clinical and Experimental Medicine (IKEM, Prague, Czech Republic; protocol #26-2010 issued by Ministry of Health of Czech Republic).

Animals and diets

Experiments were carried out in male Cyp1a1-Ren-2 transgenic rats 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), which can be easily given in the diet, the expression of the Cyp1a1 promoter is rapidly enhanced and results in a marked increase of the expression of the Ren-2 renin gene in the liver. Enhanced expression of the Ren-2 renin gene increases AngII levels.25-30 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 from Professor Mullins). All diets used in the present study were produced by Albert Weber Services to Experimental Medicine (SEMED, Prague 4, Czech Republic). Rats were fed either a rat chow without I3C (non-induced groups) or a rat chow containing 0.3% I3C (I3C-induced groups). It has been shown that I3C is a dietary supplement that does not show any harmful biological effects in transgene-negative rats, 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.25
Chemicals

Cis-4-(3-adamantan-1-yl-ureido)cyclohexyloxy) benzoic acid (c-AUCB), a sEH inhibitor, was prepared freshly and given in drinking water as described previously. The following doses of c-AUCB per 1 L of water were examined: 0.3, 3, 13, 26, 60 and 130 mg. Trandolapril (Göpren; Abbot, Prague, Czech Republic), an ACEi, was used at a dose of 6 mg/L drinking water. Our recent studies have shown that this high dose completely blocks the development of the AngII-dependent form of hypertension.

Series 1: Measurements of BP (radiotelemetry), urine collections in conscious animals, and measurement of plasma c-AUCB levels and Ren-2 renin gene expression

In accordance with the recommendation for BP measurement in experimental animals, we used a radiotelemetry system for direct BP measurements. Rats were anesthetized with a combination of tiletamine, zolazepam (Zoletil, 8 mg/kg; Virbac SA, Carros Cedex, France) and xylazine (Rometar, 4 mg/kg; Spofa, Prague, Czech Republic) intramuscularly, and TA11PA-C40 radiotelemetric probes (Data Sciences International, St. Paul, MN, USA) were implanted for direct BP measurements as described previously. Rats were allowed 10 days to recover before basal BP was recorded. Basal BP was determined for 4 days, and then induction of the renin gene was carried out until the end of the experiment that lasted 24 days.

Our previous studies have shown that after 10 days of induction a stable, severe hypertension develops. Then, the treatment with either c-AUCB or ACEi was carried out for 14 days. Untreated conscious non-induced, and I3C-induced rats and non-induced rats treated with ACEi served as controls. The animals were housed individually in metabolic cages, and their blood pressure (BP) was continuously recorded and urine was collected for 24 h. The animals were housed individually in metabolic cages, and BP measurements. Rats were anaesthetized with a combination of tiletamine, zolazepam (Zoletil, 8 mg/kg; Virbac SA, Carros Cedex, France) and xylazine (Rometar, 4 mg/kg; Spofa, Prague, Czech Republic) intramuscularly, and TA11PA-C40 radiotelemetric probes (Data Sciences International, St. Paul, MN, USA) were implanted for direct BP measurements as described previously.

Series 2: Effects of c-AUCB and ACEi in non-induced or I3C-induced Cyp1a1-Ren-2 transgenic rats on EET, DHETE, plasma and kidney tissue AngII concentrations, plasma renin activity, and plasma and kidney tissue ACE activity

It is now generally recognized that plasma and tissue AngII concentrations in anaesthetized animals are higher than those obtained from rats decapitated while conscious, and that normotensive animals show a greater increase in renin secretion in response to anaesthesia and surgery than AngII-induced hypertensive intrarenal renin-depleted animals. Therefore, in the present study we determined AngII levels in separate groups of conscious rats (as described in series 1) that were decapitated either on day 2 (to evaluate acute effects) or on day 14 (to evaluate chronic effects) after introduction of pharmacological treatment (n = 8 in each group). Plasma and whole kidney AngI and AngII concentrations were assessed by radioimmunoassay as described in detail in our previous studies. In the present study, for measurements of plasma renin activities, the commercially available RIA kit was used (Cisbio Bioassays, Paris, France); for measurements of plasma and kidney ACE activities, the commercially available RIA kit was also used (Bühlmann Laboratories, Allschwil, Switzerland). In this protocol, one unit of ACE activity was defined as the amount of enzyme required to release 1 μmol of hippuric acid per minute per liter of sample at 37°C. In addition, in the present study, ACE activities were also estimated as the ratio of AngII to AngI because we have recently found, in accordance with one

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original study in AngII-infused hypertensive rats, that in this AngII-dependent model of hypertension the ratio is a very reliable index for assessment of circulating and especially tissue ACE activity.19,26,27 The level of the arachidonic acid metabolites EET and DHETE were measured in the kidney cortex. Samples were extracted, separated by reverse-phase, high performance liquid chromatography, and analysed by negative-mode electrospray ionization and tandem mass spectroscopy as described previously.9–12 The procedure of blood sampling and the assays of individual components of RAS and EET and DHETE assays are routinely used in our laboratory, and this standardized approach allows us to compare the results to those of our previous studies evaluating the role of the RAS and CYP-dependent metabolites in the pathophysiology of the AngII-dependent form of hypertension.9,12,14,15,17,26,27

Statistical analysis
All values are expressed as means ± SEM. GraphPad Prism software (Graph Pad Software, San Diego, CA, USA) was used and, when appropriate, statistical analysis was carried out using one-way analysis of variance (ANOVA). ANOVA for repeated measurements, followed by Student–Newman–Keuls test, was carried out for the analysis within groups (e.g. before and after measurements, followed by Student–Newman–Keuls test, was carried out for the analysis within groups (e.g. before and after either I3C or pharmacological treatment). Values exceeding the 95% probability limits (P < 0.05) were considered statistically significant.

ACKNOWLEDGEMENTS
This study was principally supported by grant No. NT/12171-5 awarded by the Internal Grant Agency of the Ministry of Health to ZH. AS was supported by a Marie Curie Fellowship from the European Commission Program PEOPLE (IRG 247847). The Institute for Clinical and Experimental Medicine (IKEM) is a recipient of the project of the Ministry of Health of the Czech Republic for the development of research organization 00023001 (institutional support). SJ is supported the Grant Agency of Charles University No. 266213. The Center for Experimental Medicine (IKEM) received financial support from the European Commission within the Operational Program Prague–Competitiveness; project “Rozvoj infrastrukturny PEM” (#CZ.2.16/3.1.00/28025). JDI was supported by NIH grants HL56999 and DK38226, SHH was supported by a fellowship from the NIEHS Supported Basic Research Program. Partial support was provided by NIEHS Grant R01 ES02710, R01 ES013933 and P42 ES013933, and by West Coast Center U24 DK097154 awarded to BDH. BDH is a George and Judy Marcus Senior Fellow of the American Ashma Foundation. BDH was funded by EoCs is to develop soluble epoxide hydrolase inhibitors for treating inflammatory and neuropathic pain. The other authors declare no potential conflict of interest.

REFERENCES
Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Time-course of body weight in indole-3-carbinol (I3C)-induced and noninduced Cyp1a1-Ren-2 transgenic rats and effects c-AUCB or ACEi treatment.
Supplemental figure 1. Time-course of body weight in indole-3-carbinol (I3C)-induced and noninduced Cyp1a1-Ren-2 transgenic rats and effects c-AUCB or ACEi treatment. *P<0.05 versus baseline values. †P<0.05 versus c-AUCB- or ACEi-treated I3C-induced rats at the same time point.
Figure legends for supplemental figure.

**Supplemental figure 1.** Time-course of body weight in indole-3-carbinol (I3C)-induced and noninduced Cyp1a1-Ren-2 transgenic rats and effects c-AUCB or ACEi treatment. *P*<0.05 versus baseline values. **P**<0.05 versus c-AUCB- or ACEi-treated I3C-induced rats at the same time point.