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Inhibition of soluble epoxide hydrolase by *cis*-4-[4-(3-adamantan-1-ylureido)cyclohexyl-oxy]benzoic acid exhibits antihypertensive and cardioprotective actions in transgenic rats with angiotensin II-dependent hypertension

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ABSTRACT

The present study was undertaken to evaluate the effects of chronic treatment with *c*-AUCB {*cis*-4-[4-(3-adamantan-1-ylureido)cyclohexyl-oxy]benzoic acid}, a novel inhibitor of sEH (soluble epoxide hydrolase), which is responsible for the conversion of biologically active EETs (epoxyeicosatrienoic acids) into biologically inactive DHETEs (dihydroxyeicosatrienoic acids), on BP (blood pressure) and myocardial infarct size in male heterozygous TGR (Ren-2 renin transgenic rats) with established hypertension. Normotensive HanSD (Hannover Sprague–Dawley) rats served as controls. Myocardial ischaemia was induced by coronary artery occlusion. Systolic BP was measured in conscious animals by tail plethysmography. *c*-AUCB was administrated in drinking water. Renal and myocardial concentrations of EETs and DHETEs served as markers of internal production of epoxygenase metabolites. Chronic treatment with *c*-AUCB, which resulted in significant increases in the availability of biologically active epoxygenase metabolites in TGR (assessed as the ratio of EETs to DHETEs), was accompanied by a significant reduction in BP and a significantly reduced infarct size in TGR as compared with untreated TGR. The cardioprotective action of *c*-AUCB treatment was completely prevented by acute administration of a selective EETs antagonist [14,15-epoxyeicosa-5(*Z*)-enoic acid], supporting the notion that the improved cardiac ischaemic tolerance conferred by sEH inhibition is mediated by EETs actions at the cellular level. These findings indicate that chronic inhibition of sEH exhibits antihypertensive and cardioprotective actions in this transgenic model of angiotensin II-dependent hypertension.

Key words: angiotensin II, epoxyeicosatrienoic acid, hypertension, kidney, myocardial ischaemia/reperfusion injury, soluble epoxide hydrolase inhibitor.

Abbreviations: AA, arachidonic acid; AngII, angiotensin II; AR, area at risk; BP, blood pressure; *c*-AUCB, *cis*-4-[4-(3-adamantan-1-ylureido)cyclohexyl-oxy]benzoic acid; CYP, cytochrome P450; DHETE, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; 14,15-EEZE, 14,15-epoxyeicosa-5(*Z*)-enoic acid; FS, fractional shortening; HanSD, Hannover Sprague–Dawley; HETE, hydroxyeicosatetraenoic acid; HR, heart rate; IA, infarct area; I/R, ischaemia/reperfusion; LV, left ventricular; LVH, left ventricular hypertrophy; LVW, left ventricular weight; PVC, premature ventricular complex; RAS, renin–angiotensin system; SBP, systolic BP; sEH, soluble epoxide hydrolase; *t*-AUCB, *trans*-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid; TGR, Ren-2 renin transgenic rat(s).

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INTRODUCTION

Ischaemic heart disease is the leading cause of mortality in industrialized nations [1]. Hypertension and hypertension-induced LVH [LV (left ventricular) hypertrophy] have been recognized in the Framingham cohort study as independent risk factors of myocardial I/R (ischaemia/reperfusion) injury [2]. Therefore new therapeutic strategies for the treatment of hypertension and for the protection of the myocardium against I/R injury have been studied [3–9].

An increasing body of evidence indicates that CYP (cytochrome P450)-dependent metabolites of AA (arachidonic acid), such as the EETs (epoxyeicosatrienoic acids), play an important role in the regulation of vascular tone and extracellular fluid volume homoeostasis [10–12]. Recent studies have demonstrated that a reduced availability of biologically active EETs as the result of increased conversion of EETs into biologically inactive DHETEs (dihydroxyeicosatrienoic acids) significantly contributes to the pathophysiology of hypertension in the TGR (Ren-2 renin transgenic rat) strain [13,14]. The TGR represents a unique AngII (angiotensin II)-dependent form of hypertension in which the development of hypertension is related to the insertion of a mouse *Ren-2* renin gene into the genome of normotensive HanSD (Hannover Sprague–Dawley) rats [15].

Recent studies have provided evidence that EETs are cardioprotective in several models of I/R injury [9,16–19]. However, the effects of chronic inhibition of sEH (soluble epoxide hydrolase), an enzyme responsible for the conversion of EETs into DHETEs, on BP (blood pressure) and myocardial resistance to I/R have not been evaluated in TGR. We hypothesized that treatment with an sEH inhibitor in this monogenic model of AngII-dependent hypertension will exhibit antihypertensive and cardioprotective actions. Therefore the aim of the study was to characterize the effects of treatment with the novel sEH inhibitor *c*-AUCB {*cis*-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid} on the BP, myocardial infarct size and the incidence and severity of ischaemic and reperfusion arrhythmias in normotensive HanSD rats and hypertensive TGR. Finally, to gain a more detailed insight into the potential cardioprotective role of EETs after sEH inhibition, we evaluated if acute administration of a selective EETs antagonist can abolish the cardioprotective effect of chronic *c*-AUCB treatment.

MATERIALS AND METHODS

Animals

All animals used in the present study were bred at the Center for Experimental Medicine from stock animals supplied from Max Delbrück Center for Molecular Medicine, Berlin, Germany (a gift from Dr Michael Bader and Dr Detlev Ganten). The TGR strain was constructed by inserting the mouse *Ren-2* renin gene, including 5 kb of 5′-flanking sequences and 9 kb of 3′-flanking sequences, into the rat genome of HanSD rats. Heterozygous TGR were generated by breeding male homozygous TGR with female homozygous HanSD rats as described previously [15]. Animals were fed with a standard rat chow containing 0.4 % NaCl (SEMED) with free access to tap water.

The studies were carried out in accordance with guidelines and practices established by the Institute for Clinical and Experimental Medicine Animal Care and Use Committee.

Chemicals

The sEH inhibitor *c*-AUCB was given in drinking water prepared freshly as described previously [20,21] at the dose of 13 mg/l. Briefly, crystalline *c*-AUCB (13 mg) was dissolved in ethanol (5 ml) and cyclodextrin (150 mg) after 5 min of sonication and this solution was added to 1 litre of water. Hydrogen carbonate (3 ml/l) was added to ensure that the water did not become acidic since low pH can cause the compound to precipitate. This dose of *c*-AUCB was used in our recent studies and we found that it exhibited significant antihypertensive actions and substantially increased tissue concentrations of EETs [21]. We selected *c*-AUCB for the work to be consistent with our earlier studies [11,20,21]. At the time these studies were initiated, synthetic methods were less difficult for the *cis*-isomer, *c*-AUCB, compared with the *trans*-isomer, *trans*-4-[4-(3-adamantan-1-yl-ureido)cyclohexyloxy]benzoic acid (referred to as *t*-AUCB hereafter). In addition, while the potency and physical properties of the two isomers are similar [20], *c*-AUCB showed slightly better pharmacokinetic properties in rats than *t*-AUCB. A comparison of these pharmacokinetic properties is provided in the Supplementary online data (at http://www.clinsci.org/cs/122/cs1220513add.htm).

The putative selective EETs antagonist, 14,15-EEZE [14,15-epoxyeicosa-5(*Z*)-enoic acid; Cayman Chemical], was given into the right jugular vein at the dose of 250 μg/5 min before the onset of the coronary artery occlusion as described below in experimental series 2. This dose of 14,15-EEZE was selected on the basis of previous studies that demonstrated that it prevented the cardioprotection induced by either exogenous or endogenous EETs in the canine heart [16].

Experimental design

Series 1: effects of *c*-AUCB on BP and urinary excretion of EETs/DHETEs

In this series, heterozygous male TGR with established hypertension and male HanSD rats were employed. The following experimental groups were examined:

(i) TGR + vehicle ($n = 5$), (ii) HanSD + vehicle ($n = 5$), (iii) TGR + *c*-AUCB ($n = 6$) and (iv) HanSD + *c*-AUCB ($n = 6$).

Animals were randomly divided into the aforementioned experimental groups and remained untreated from weaning until 99 days of age. SBP (systolic BP) was measured every other day in appropriately trained conscious animals by tail plethysmography via a tail-cuff apparatus (MC 4000; Hatteras Instruments) from 88 days of age onwards. On each occasion SBP was determined as the average of four measurements. A close correlation between measurements by tail plethysmography and direct BP measurements with an indwelling catheter was found with this method, which is regularly employed in our laboratory [14,22]. At the age of 100 days an appropriate treatment was started for 48 h. At 98 and 102 days of age (prior to and after treatment initiation) animals were placed in metabolic cages for a 12 h urine collection. Urine samples were stored at $-80\,^{\circ}C$ until assay. Urinary concentrations of EETs and DHETEs were measured by ELISA (Detroit R&D) according to the manufacturer's instructions using commercially available kits as verified in our previous study [23]. At the end of the experiment, rats were killed by overdosing with sodium pentobarbital (Sanofi-Sante Animale) and the LVW (LV weight)/TL (tibia length) ratio was calculated to evaluate the degree of cardiac hypertrophy [21].

Series 2: effects of *c*-AUCB and 14,15-EEZE on myocardial infarct size and the incidence and severity of ventricular arrhythmias induced by I/R

In this series, TGR and HanSD rats were subjected to the same treatment protocol as described in series 1, i.e. animals were exposed to the *c*-AUCB treatment for 48 h prior to initiating the coronary artery occlusion. Studies were performed in the following experimental groups: (i) TGR + vehicle ($n = 11$), (ii) HanSD + vehicle ($n = 8$), (iii) TGR + *c*-AUCB ($n = 12$), (iv) HanSD + *c*-AUCB ($n = 10$), (v) TGR *c*-AUCB + 14,15-EEZE ($n = 9$) and (vi) HanSD + *c*-AUCB + 14,15-EEZE ($n = 10$).

Rats were subjected to regional myocardial I/R as described previously using an open-chest model [24]. Animals were anaesthetized with sodium pentobarbital (60 mg/kg of body weight, intraperitoneal). A heparinized cannula was placed in the left carotid artery for BP monitoring with a pressure transducer (P23Gb; Gould Instruments Systems) and the data were subsequently analysed by our custom-designed software. Rats were ventilated with room air at 68 strokes/min (tidal volume, 1.2 ml/100 g of body weight) using a rodent ventilator (Ugo Basile). HR (heart rate) was derived from the BP curve. The rectal temperature was maintained between 36.5 and $37.5\,^{\circ}C$ by a servo-controlled heated table throughout the experiment. A left thoracotomy was performed, and a silk suture 5-0 (Chirmax) was placed loosely around the LAD (left anterior descending) coronary artery 1–2 mm distal to its origin. After 10 min of stabilization, regional ischaemia was induced by tightening of the suture threaded through a polyethylene tube. After a 20 min occlusion period, the ligature was released and reperfusion of previously ischaemic tissue continued for 3 h.

Determination of the infarct size and arrhythmias

Then, hearts were excised and washed with saline through the aorta. The IA (infarct area) and the AR (area at risk) were delineated as described previously [24] by staining with potassium permanganate and 2,3,5-triphenyltetrazolium chloride (Sigma–Aldrich) respectively. Hearts were cut perpendicularly to the long axis of the ventricle into the 1-mm-thick slices and stored overnight in formaldehyde solution. The size of the IA and the size of the AR were determined by computerized planimetry and the IA was normalized to the AR (IA/AR). The size of the left ventricle was determined and the AR was normalized to the left ventricle (AR/LV ratio). This approach has been validated in many experimental studies and is currently accepted as the 'gold standard' for the measurement of myocardial infarct size in small animals [6].

Ventricular arrhythmias during the ischaemic insult and during early reperfusion were assessed according to the Lambeth Conventions [25]. Briefly, PVCs (premature ventricular complexes) occurring as singles, salvos (two or three PVCs) or tachycardia (a run of four or more consecutive PVCs) were counted separately. The incidence of ventricular tachycardia and fibrillation was also evaluated. The severity of arrhythmias in each group was evaluated by means of a 5-point arrhythmia score as described elsewhere [26].

Series 3: effects of *c*-AUCB on AngII concentrations

Animals were divided into the following experimental groups and were exposed to the same treatment protocol as in series 1: (i) TGR + vehicle ($n = 8$), (ii) HanSD + vehicle ($n = 7$), (iii) TGR + *c*-AUCB ($n = 8$) and (iv) HanSD + *c*-AUCB ($n = 7$).

Since it is now recognized that plasma and tissue AngII concentrations in anaesthetized animals are higher than those obtained from decapitated conscious rats and that normotensive animals exhibit greater increases in renin secretion in response to anaesthesia and surgery than AngII-dependent hypertensive intrarenal renin-depleted animals [22], in the present study rats from each experimental group were killed by decapitation and plasma and tissue were collected. Plasma, whole kidney and LV myocardial AngII levels were measured by RIA as described previously [22]. This approach for blood and tissue sampling and AngII assay, which is routinely used in our laboratory, allows comparison of the present results with those of our previous studies evaluating

the role of the RAS (renin–angiotensin system) in the pathophysiology of hypertension and tissue damage [14,21–23].

Series 4: effects of *c*-AUCB and 14,15-EEZE treatment on EETs, DHETEs and HETEs (hydroxyeicosatetraenoic acids) concentrations

Animals were divided into the following experimental groups and were exposed to the same chronic treatment protocol as in series 1 and acute pretreatment with 14,15-EEZE as in series 2: (i) TGR + vehicle ($n = 8$), (ii) HanSD + vehicle ($n = 7$), (iii) TGR + *c*-AUCB ($n = 8$), (iv) HanSD + *c*-AUCB ($n = 7$), (v) TGR *c*-AUCB + 14,15-EEZE ($n = 8$) and (vi) HanSD + *c*-AUCB + 14,15-EEZE ($n = 8$).

At 5 min after intrajugular acute administration of either 500 μl of saline vehicle (groups 1–4) or 250 μg of 14,15-EEZE (dissolved in the same volume), rats were killed with an excess dose of intravenous pentobarbital sodium and tissues collected. The levels of the AA metabolites, EETs, DHETEs and HETEs were measured in the kidney cortex and LV myocardium. Samples were extracted, separated by reverse-phase HPLC and analysed by negative-mode electrospray ionization and tandem MS as described previously [21]. Specifically, 5,6-EETs, 8,9-EETs, 11,12-EETs and 14,15-EETs and DHETEs respectively were measured separately and then pooled together for the presentation. Results are shown as total concentrations of EETs and DHETEs respectively, because it is well recognized that these metabolites are the most biologically active products formed in the CYP epoxygenase enzymatic pathway. Similarly, 5-HETEs, 8-HETEs, 9-HETEs, 11-HETEs, 12-HETEs, 15-HETEs, 19-HETEs and 20-HETEs respectively were analysed and then again pooled, since it is recognized that these metabolites are the most biologically active products of the CYP hydroxylase enzymatic pathway [27,28].

Series 5: effects of *c*-AUCB on basal cardiac function assessed by echocardiography and invasive technique

Animals were divided into the following experimental groups and were exposed to the same chronic treatment protocol as in series 1: (i) TGR + vehicle ($n = 9$), (ii) HanSD + vehicle ($n = 9$), (iii) TGR + *c*-AUCB ($n = 9$) and (iv) HanSD + *c*-AUCB ($n = 8$).

Evaluation of geometrical and functional parameters of the left ventricle was performed using GE Vingmed System with 14 MHz linear matrix probe and method established in our laboratory [29,30]. Animals were anaesthetized by inhalation of 2% isoflurane. For baseline echocardiographic evaluation, the following diastolic and systolic dimensions of the left ventricle were measured: posterior and anterior wall thickness and cavity diameters. From these dimensions, the main functional parameter, FS (fractional shortening), was derived by the following equation:

$$\text{FS}\,(\%) = (\text{LV cavity diameter in diastole} - \text{LV cavity diameter in systole}/\text{LV cavity diameter in diastole})$$

After echocardiographic examination, LV function was assessed invasively using a 2F catheter-tip manometer (Millar Instruments) introduced into the LV cavity via the right carotid artery as described previously [31]. The following parameters were derived from the pressure curve: peak systolic pressure, end-diastolic pressure, maximum rates of pressure rise and fall [$+(dP/dt)_{max}$ and $-(dP/dt)_{max}$ respectively], time constant of relaxation (τ) and HR.

Statistical analysis

All values are expressed as means ± S.E.M. GraphPad Prism software was used and statistical analysis was performed using the Student's *t* test, Wilcoxon's signed-rank test for unpaired data or one-way ANOVA where appropriate. ANOVA for repeated measurements, followed by the Student–Newman–Keuls test was performed for the analysis within groups (e.g. before and after *c*-AUCB administration). Differences in the number of ventricular arrhythmias between the groups were evaluated by the Mann–Whitney *U* test. The incidence of tachycardia and fibrillation was examined by the Fischer's exact test. Values exceeding the 95% probability limits ($P < 0.05$) were considered statistically significant.

RESULTS

Series 1: effects of *c*-AUCB treatment on BP and urinary excretion of EETs/DHETEs

As shown in Figure 1(A), TGR rats before the beginning of treatment are markedly hypertensive (198 ± 4 mmHg) and the treatment with *c*-AUCB resulted in significant decreases in SBP (to 179 ± 3 mmHg, $P < 0.05$ compared with initial values). SBP in HanSD remained within the normotensive range throughout the study (from 128 ± 3 to 131 ± 3 mmHg). Treatment with *c*-AUCB had no effect on SBP in HanSD rats. Untreated TGR exhibited severe LVH (measured as index of LVW/TL) as compared with untreated HanSD rats (30.6 ± 1.1 compared with 22.4 ± 0.9, $P < 0.05$), which was not altered by the treatment with *c*-AUCB.

Figure 1(B) summarizes the availability of biologically active epoxygenase metabolites when expressed as the ratio of EETs to DHETEs. The basal ratio (before treatment with *c*-AUCB) was significantly lower in TGR than in HanSD rats (1.09 ± 0.15 compared with 2.79 ± 0.13, $P < 0.05$). The treatment with *c*-AUCB significantly increased this ratio in TGR as well in HanSD rats to 2.37 ± 0.29 and 3.28 ± 0.13 respectively when compared with basal values ($P < 0.05$ in both

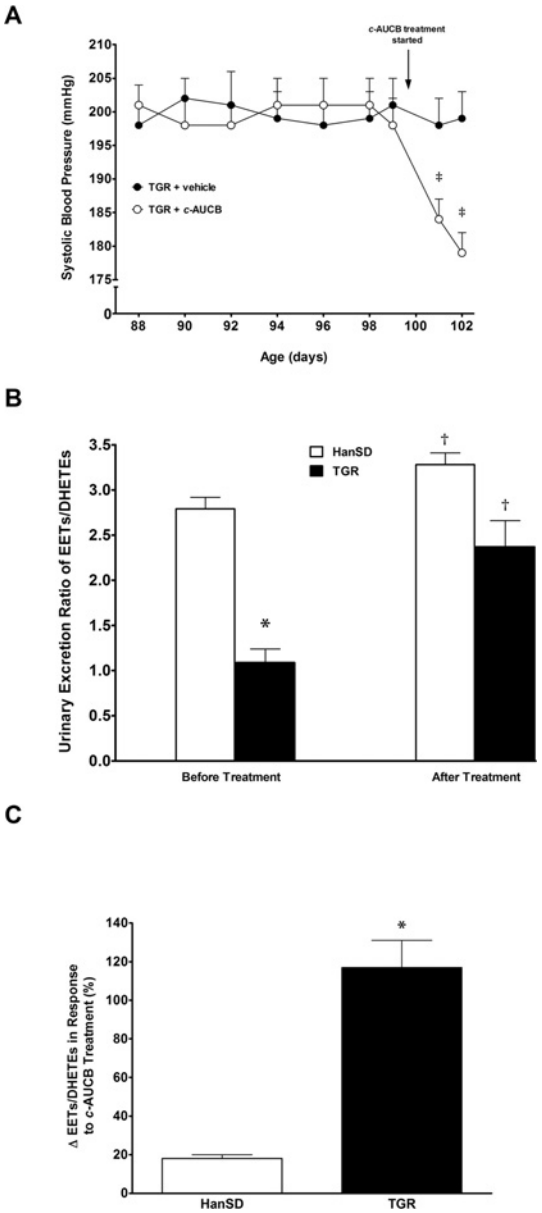

Figure 1 Effect on SBP (A), urinary excretion ratio of EETs and DHETEs (B) and the change in the urinary excretion ratio of EETs and DHETEs (C) in HanSD and TGR before and after treatment with *c*-AUCB

(A) Changes in SBP in TGR either untreated (vehicle) or treated with sEH inhibitor (*c*-AUCB). (B) Urinary excretion ratio of EETs to DHETEs in HanSD rats and TGR before and after treatment with *c*-AUCB. (C) Changes in urinary excretion ratio of EETs to DHETEs in HanSD rats and TGR in response to *c*-AUCB administration. Results shown are means $\pm$ S.E.M. $^{*}P < 0.05$ compared with corresponding HanSD; $\dagger P < 0.05$ compared with the corresponding untreated group; $\ddagger P < 0.05$ compared with values before treatment.

strains). However, this increase in the EETs/DHETEs ratio was markedly higher in TGR than in HanSD rats (Figure 1C).

Series 2: effects of *c*-AUCB and 14,15-EEZE on myocardial infarct size and the incidence and severity of ventricular arrhythmias induced by I/R

As shown in Figure 2(A), the normalized AR (expressed as the AR/LV ratio) was between 35 and 41% and did not significantly differ among experimental groups. As shown in Figure 2(B), myocardial infarct size normalized to the AR was significantly lower in untreated TGR than in untreated HanSD rats (58.1 ± 2.2% compared with 75.6 ± 3.2%, $P < 0.05$). Treatment with *c*-AUCB did not alter the infarct size in HanSD rats, but further protected TGR when compared with untreated TGR (43.3 ± 1.8% compared with 58.1 ± 2.2%, $P < 0.05$). The acute pretreatment with 14,15-EEZE completely abolished the protective effect of *c*-AUCB treatment on the infarct size in TGR (Figure 2B).

There were no significant differences in the incidence and severity of ischaemic and reperfusion ventricular arrhythmias between untreated TGR and HanSD rats. Treatment with either *c*-AUCB alone or the combination of *c*-AUCB and acute pretreatment with 14,15-EEZE did not significantly alter arrhythmias in TGR or HanSD rats (results not shown).

Series 3: effects of *c*-AUCB treatment on AngII concentrations

As shown in Figure 3, plasma, kidney and LV myocardial AngII levels were significantly higher in untreated TGR than in HanSD rats (22 ± 2 compared with 12 ± 2 fmol/ml and 173 ± 7 compared with 62 ± 5 and 16 ± 2 compared with 9 ± 2 fmol/g of tissue respectively, $P < 0.05$ in each case). In both TGR and HanSD rats, treatment with *c*-AUCB did not affect AngII levels in plasma, kidney or myocardium (Figure 3).

Series 4: effects of *c*-AUCB and 14,15-EEZE treatment on EETs, DHETEs and HETEs concentrations

As shown in Figure 4(A), untreated TGR revealed significantly lower renal concentrations of EETs than HanSD rats (641 ± 36 compared with 781 ± 34 ng/g of tissue, $P < 0.05$); treatment with *c*-AUCB resulted in significant increases in renal concentrations of EETs in TGR as well as in HanSD rats. Although untreated TGR exhibited a tendency towards increased renal concentrations of DHETEs as compared with HanSD rats, it did not reach statistical significance (Figure 4B). Treatment with *c*-AUCB caused significant decreases in renal concentrations of DHETEs in TGR as well as in HanSD rats, but the decreases in DHETEs were more pronounced in TGR. Figure 4(C) summarizes the results on the intrarenal availability of biologically active epoxygenase metabolites expressed as

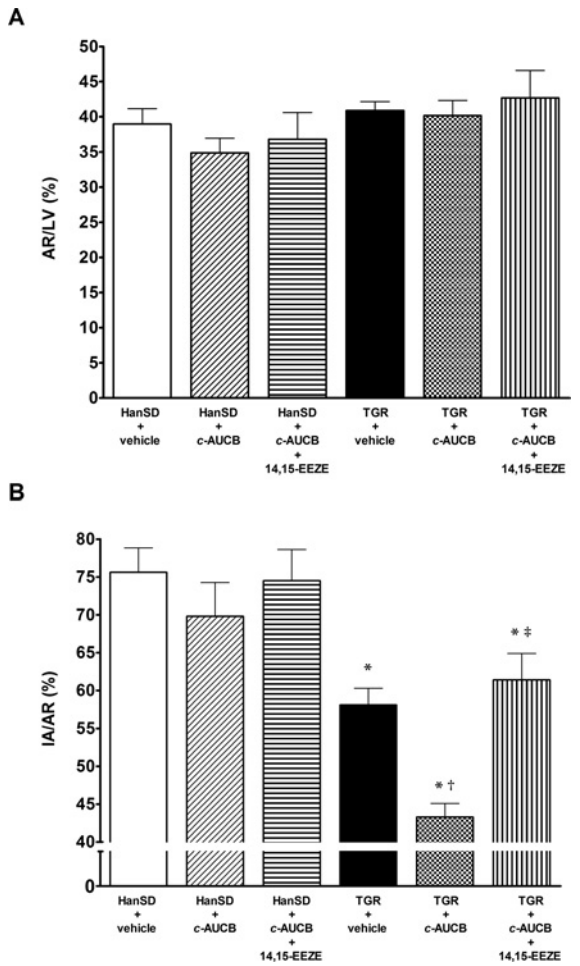

Figure 2 Effect of *c*-AUCB or a combination of *c*-AUCB and a selective EETs antagonist on myocardial AR and infacrt size

(A) Myocardial AR normalized to the size of left ventricle (AR/LV) and (B) infarct size (IA) expressed as a percentage of the AR (IA/AR) in HanSD rats and TGR either untreated (vehicle), treated with *c*-AUCB or a combination of *c*-AUCB and a selective EETs antagonist (14,15-EEZE). Results shown are means ± S.E.M. $^{*}P < 0.05$ compared with corresponding HanSD; †$P < 0.05$ compared with untreated TGR.

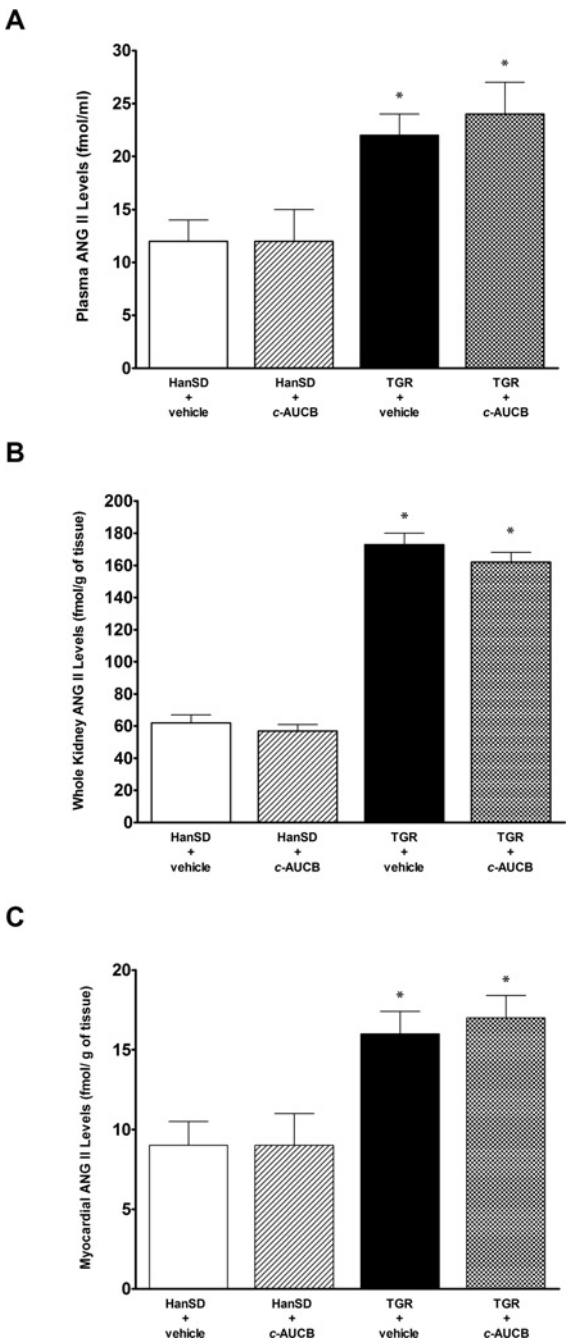

Figure 3 Effect of *c*-AUCB on plasma (A), kidney (B) and LV myocardial (C) AngII concentrations in HanSD rats and TGR

Plasma (A), kidney (B) and LV myocardial (C) concentrations of AngII in HanSD rats and TGR either untreated (vehicle) or treated with *c*-AUCB are shown. Results shown are means ± S.E.M. $^{*}P < 0.05$ compared with corresponding HanSD.

the EETs/DHETEs ratio. This ratio was markedly lower in untreated TGR than in HanSD rats. Treatment with *c*-AUCB caused significant increases in this ratio in TGR as well in HanSD rats. However, as shown in Figure 4(D), this increase in the EETs/DHETEs ratio was markedly higher in TGR than in HanSD rats (215 ± 9 % compared with 44 ± 4 %, $P < 0.05$). The acute pretreatment with 14,15-EEZE did not alter renal concentrations of EETs and DHETEs in *c*-AUCB-treated TGR or in HanSD rats.

As shown in Figure 5(A), myocardial levels of EETs in untreated TGR were significantly lower than in HanSD rats (335 ± 22 compared with 401 ± 36 ng/g of tissue, $P < 0.05$). Treatment with *c*-AUCB did not change myocardial levels of EETs in HanSD rats, but significantly increased EETs concentrations in TGR. Likewise with renal concentrations of DHETEs, there were no significant differences in myocardial concentrations in untreated TGR and HanSD rats, and treatment with *c*-AUCB resulted in significant decreases of DHETEs in TGR as well as HanSD rats (Figure 5B). Figure 5(C) summarizes the results

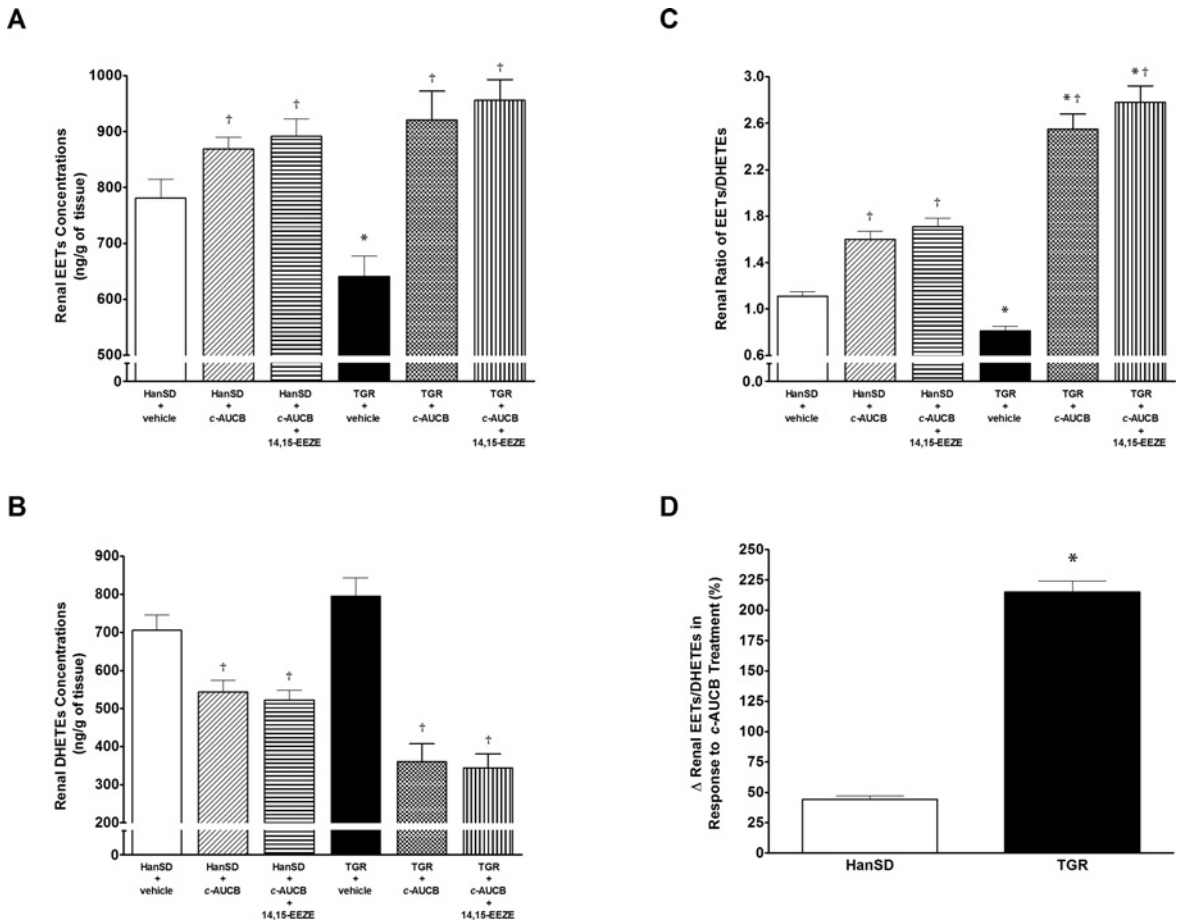

Figure 4 Effect of *c*-AUCB or a combination of *c*-AUCB and a selective EETs antagonist on EETs, DHETEs, the ratio of EETs to DHETEs and changes in the ratio of EETs to DHETEs in the kidney cortex of HanSD rats and TGR

EETs (A), DHETEs (B), the ratio of EETs to DHETEs (C) and changes in the ratio of EETs to DHETEs (D) in the kidney cortex of HanSD rats and TGR that were untreated (vehicle), treated with *c*-AUCB or treated with a combination of *c*-AUCB and a selective EETs antagonist (14,15-EEZE) are shown. Results shown are means ± S.E.M. $^{*}P < 0.05$ compared with corresponding HanSD. †$P < 0.05$ compared with corresponding untreated group.

on the availability of biologically active epoxygenase metabolites in the myocardium when expressed as the EETs/DHETEs ratio. This ratio was significantly lower in untreated TGR than in HanSD rats (3.81 ± 0.19 compared with 5.28 ± 0.24, $P < 0.05$). Treatment with *c*-AUCB significantly increased this ratio in TGR as well in HanSD rats. However, as shown in Figure 5(D), this increase in myocardial EETs/DHETEs ratio was substantially greater in TGR than in HanSD rats (386 ± 21 % compared with 95 ± 6 %, $P < 0.05$). Similar to the kidney, the acute pretreatment with 14,15-EEZE did not alter EETs or DHETEs concentrations in the myocardium in *c*-AUCB-treated TGR or HanSD rats.

Data in Figure 6 demonstrate that there were no significant differences in the intrarenal and myocardial availability of biologically active hydroxylase metabolites in untreated TGR and HanSD rats. Treatment with *c*-AUCB alone or the combination of *c*-AUCB and acute pretreatment with 14,15-EEZE did not change tissue HETEs concentrations in any of the experimental groups.

Series 5: effects of *c*-AUCB on basal cardiac function

As summarized in Table 1, untreated TGR exhibited signs of LVH associated with marked elevations of LV peak systolic pressure and dP/dt as compared with HanSD rats. However, other basal parameters of LV function (such as FS, end-diastolic pressure, constant τ, HR etc.) in untreated TGR were not significantly altered as compared with HanSD rats. The treatment with *c*-AUCB did not modify any cardiac functional parameters in HanSD rats. In TGR the administration of *c*-AUCB for 48 h significantly reduced LV peak systolic pressure and dP/dt as compared with untreated TGR and exhibited a tendency to lower parameters reflecting LVH. All other

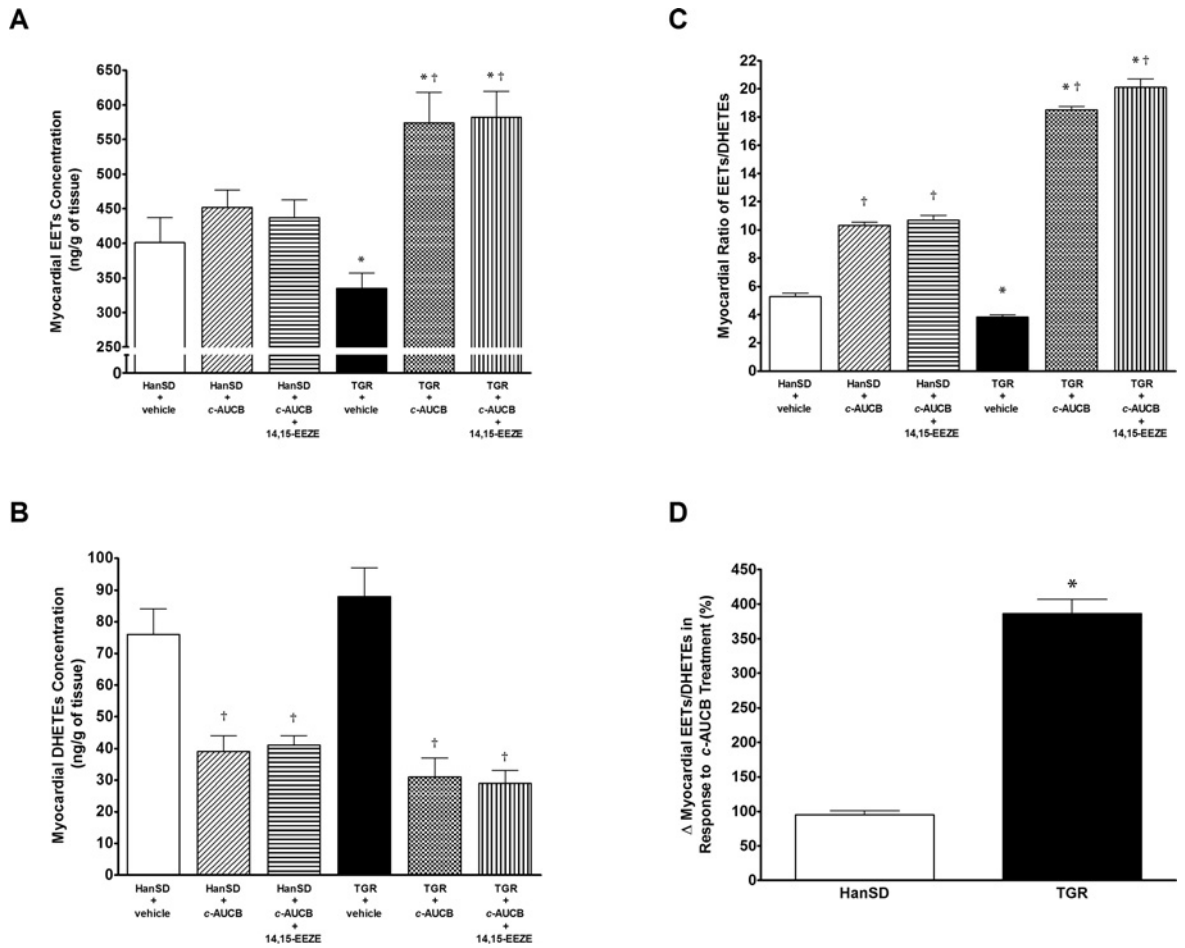

Figure 5 Effect of *c*-AUCB or a combination of *c*-AUCB and a selective EETs antagonist on EETs, DHETEs, the ratio of EETs to DHETEs and changes in the ratio of EETs to DHETEs in the LV myocardium of HanSD rats and TGR

EETs (A), DHETEs (B), the ratio of EETs to DHETEs (C) and changes in the ratio of EETs to DHETEs (D) in LV myocardium of HanSD rats and TGR that were untreated (vehicle), treated with *c*-AUCB or treated with a combination of *c*-AUCB and a selective EETs antagonist (14,15-EEZE) are shown. $^{*}P < 0.05$ compared with corresponding HanSD. Results shown are means $\pm$ S.E.M. $\dagger P < 0.05$ compared with corresponding untreated group.

cardiac functional parameters were not modified by the *c*-AUCB treatment.

DISCUSSION

The first major finding of the present study is that treatment with the novel sEH inhibitor *c*-AUCB resulted in significant decreases in BP in TGR, which was associated with significant increases in the availability of biologically active epoxygenase metabolites assessed as the ratio of EETs to DHETEs. In addition, our results show that chronic inhibition of sEH elicited markedly higher increases in the ratio of EETs to DHETEs in TGR than in HanSD rats. These data are in good agreement with our recent findings showing that TGR in the phase of established hypertension exhibit a markedly reduced availability of biologically active EETs in comparison with age-matched normotensive HanSD rats as the consequence of its enhanced conversion into biologically inactive DHETEs [13,14]. Our present data strongly support the recently proposed notion that the deficiency in biologically active EETs represents a permissive factor in the development and maintenance of hypertension in TGR [13,14]. The critically important issue of our present finding is related to the following question: what are the underlying mechanism(s) responsible for the antihypertensive actions of chronic sEH inhibition in TGR?

In this regard, it is important to realize that studies performed during the last three decades have revealed that EETs exhibit biologically important effects on the regulation of renal tubular transport of sodium and on the regulation of vascular tone [10–12,27]. At the kidney level it has been shown that EETs inhibit sodium reabsorption in the renal proximal tubule by blocking

Table 1 LV basal functional parameters evaluated by echocardiography and invasive technique in untreated and *c*-AUCB-treated HanSD rats and TGR

Values are means ± S.E.M. $^{*}P < 0.05$ compared with HanSD rats; †$P < 0.05$ compared with untreated TGR. $+(dP/dt)_{max}$, maximum rate of pressure rise; $-(dP/dt)_{max}$, maximum rate of pressure fall; τ, time constant of relaxation.

Parameter	HanSD + vehicle	HanSD + *c*-AUCB	TGR + vehicle	TGR + *c*-AUCB
Echocardiography				
Diastolic diameter (mm)	7.72 ± 0.24	7.98 ± 0.12	7.99 ± 0.08	8.13 ± 0.13
Systolic diameter (mm)	4.25 ± 0.25	4.48 ± 0.09	4.53 ± 0.06	4.67 ± 0.14
Anterior wall thickness in diastole (mm)	1.71 ± 0.04	1.72 ± 0.02	2.01 ± 0.06*	1.93 ± 0.07*
Posterior wall thickness in diastole (mm)	1.92 ± 0.03	1.91 ± 0.03	2.21 ± 0.07*	2.11 ± 0.06*
Anterior wall thickness in systole (mm)	2.84 ± 0.06	2.90 ± 0.03	3.37 ± 0.09*	3.24 ± 0.05*
Posterior wall thickness in systole (mm)	3.02 ± 0.09	3.11 ± 0.05	3.41 ± 0.06	3.21 ± 0.07
FS (%)	45.2 ± 1.6	43.9 ± 0.5	43.1 ± 1.1	42.7 ± 0.9
Invasive technique				
Peak SBP (mmHg)	120 ± 2	121 ± 5	165 ± 3*	150 ± 5*†
$+(dP/dt)_{max}$ (mmHg/s)	8311 ± 212	8592 ± 229	9993 ± 195*	9146 ± 218*†
$-(dP/dt)_{max}$ (mmHg/s)	7354 ± 184	7239 ± 120	9706 ± 200*	8605 ± 305*†
End-diastolic pressure (mmHg)	5.6 ± 0.9	5.1 ± 0.8	5.9 ± 0.3	6.5 ± 0.6
Constant τ (ms)	12.7 ± 0.2	13.2 ± 0.2	13.3 ± 0.3	13.8 ± 0.3
HR (beats/min)	342 ± 11	331 ± 7	355 ± 10	326 ± 7

the sodium–hydrogen exchanger [32] and also decrease sodium reabsorption in the cortical collecting duct by blocking the epithelial sodium channels [33].

Most of the available evidence indicates that the EETs' antihypertensive properties are mainly associated with their action on sodium excretion [10–12,27]. It is therefore conceivable that net intrarenal deficiency of EETs in the kidney of TGR contributes to the impairment of the pressure–natriuresis relationship and, consequently, to the maintenance of hypertension in this model. Thus chronic treatment with the sEH inhibitor increased the intrarenal availability of biologically active epoxygenase metabolites in TGR (assessed as the ratio of intrarenal EETs to DHETEs) to levels even greater than those observed in HanSD rats. Therefore, on the basis of this knowledge and on our present findings, we propose that the first potential mechanism underlying the antihypertensive actions of the chronic sEH inhibition could be an improvement of the impaired pressure-natriuresis in TGR.

At the level of the vasculature it has been shown that EETs elicit vasodilation, and it has also been demonstrated that EETs oppose the vasoconstrictor action of AngII [34]. On the basis of these results, we suggest that the second potential mechanism of the antihypertensive actions of chronic sEH inhibition in TGR could be EETs-mediated attenuation of the previously well-documented selectively enhanced peripheral and renal vascular responsiveness to AngII [13,14]. This would decrease peripheral and renal vascular resistance and thereby attenuate the hypertension in TGR.

Collectively, with the above-discussed observations in mind we suggest that the underlying mechanism(s) responsible for the antihypertensive actions of sEH inhibitor in TGR is the combination of actions of enhanced availability of biologically active epoxygenase metabolites at the level of the kidney and the vasculature.

The second major finding of the present study is that the myocardial infarct size induced by acute I/R insult was significantly smaller in TGR than in HanSD rats. On the other hand, the incidence and severity of ischaemic and reperfusion arrhythmias were not significantly different. These findings indicate that cardiac tolerance to irreversible I/R injury is enhanced in TGR as compared with normotensive HanSD rats.

This notion is of great interest, because for the last 35 years it was believed that the hypertrophic myocardium exhibits a decreased tolerance to I/R injury, and indeed the clinical phenomenon of 'stone heart' during cardiothoracic surgery was first described in the hypertrophic heart [35]. In addition, a number of experimental studies using various methods of induction of LVH and different endpoints of myocardial injury have revealed and confirmed the original clinical observation that the hypertrophic myocardium is more vulnerable to I/R than the normal one [36–38]. We cannot offer a fully satisfactory explanation of our results. It is possible that differences in the systemic and cardiac tissue activities of RAS between TGR and HanSD rats might be responsible for our finding. However, it is also important to emphasize that basal LV functional parameters assessed by echocardiography and by invasive technique did not significantly differ between TGR and

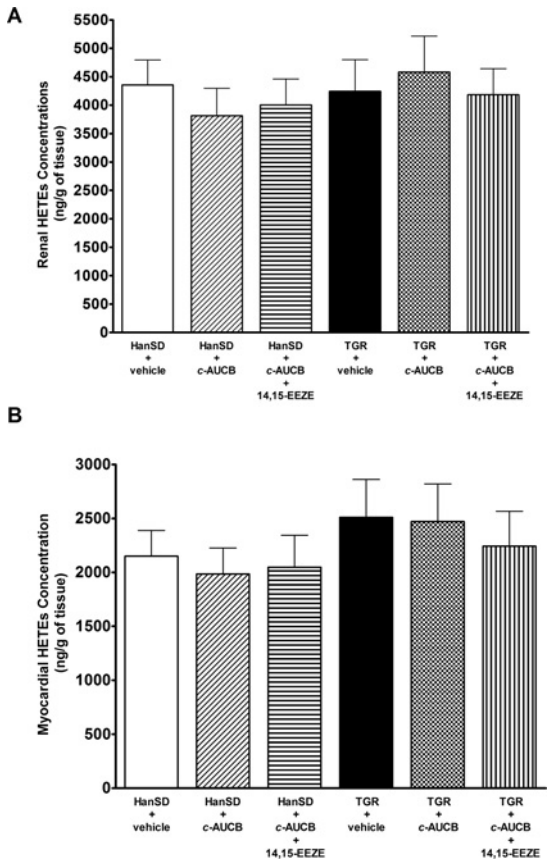

Figure 6 Effect of *c*-AUCB or a combination of *c*-AUCB and a selective EETs antagonist on HETEs in the kidney cortex and LV myocardium of HanSD rats and TGR

Concentrations of HETEs in the kidney cortex (A) and in the LV myocardium (B) of HanSD rats and TGR that were untreated (vehicle), treated with *c*-AUCB or treated with a combination of *c*-AUCB and a selective EETs antagonist (14,15-EEZE) are shown. Results shown are means ± S.E.M.

HanSD rats, except for systolic pressure and d*P*/d*t*. It is evident that the insertion of *Ren-2* renin gene itself did not impair cardiac function and therefore the improved myocardial tolerance to I/R injury in TGR cannot be ascribed to alterations in cardiac performance.

In this context it is important to note that not all studies evaluating infarct size as the primary endpoint of I/R injury in hypertensive animals with LVH have unequivocally demonstrated an enhanced infarct size. On the one hand, there are studies showing that the mortality and infarct size are increased in hypertensive animals with LVH [5,36–38]. On the other hand, several studies using different genetic and experimental models of hypertension show that the infarct size was either not significantly different or was even reduced in hypertensive animals as compared with normotensive controls [39,40]. Similarly, rats with moderate systemic hypertension and LVH induced by chronic intermittent hypoxia also exhibit smaller infarct size induced by I/R insult than normoxic (normotensive) controls [24]. In view of this information, our present finding that the infarct size in TGR was lower than in HanSD rats appears less controversial. Our data do not allow us to delineate the underlying mechanism(s) responsible for the higher ischaemic tolerance in hypertrophic hearts of TGR and future studies are needed to address this issue.

Our third major finding is that the novel sEH inhibitor *c*-AUCB significantly reduced infarct size in TGR. This finding supports the notion that the normalization of availability of biologically active EETs in TGR is cardioprotective. This notion is strongly supported by our observation that this cardioprotective effect of *c*-AUCB treatment in TGR was abolished by pretreatment with a putative selective EETs antagonist, 14,15-EEZE, which did not alter availability of EETs. This is in good agreement with recent studies showing that administration of EETs reduced infarct size in dogs and mice [16–19]. Another recent study by Chaudhary et al. [41] also demonstrated that perfusion of isolated murine hearts with *t*-AUCB, another newly developed sEH inhibitor, significantly improved the post-ischaemic recovery of contractile function and reduced infarct size.

Taken together, our present results are in line with earlier findings and extend previous observations demonstrating that chronic pharmacological inhibition of sEH activity in an AngII-dependent model of hypertension results in a marked increase in biological availability of EETs, and their actions in the myocardium subsequently result in enhanced cardiac ischaemic tolerance. However, our present data do not allow us to determine the specific underlying mechanism(s) responsible for the cardioprotective actions of sEH inhibition and it is therefore obvious that additional studies are needed to reveal mechanism(s) responsible for the cardioprotection conferred by increased availability of biologically active epoxygenase metabolites at the cellular and subcellular levels in TGR.

In view of our findings in TGR and of the results of marked cardioprotective actions in previous studies in normotensive rats, mice and healthy dogs with either exogenously infused EETs or after the administration of an sEH inhibitor [9,16–19], our present finding that chronic treatment with the novel sEH inhibitor *c*-AUCB did not reduce infarct size in transgene-negative normotensive HanSD rats is of interest. Reasons for the lack of cardioprotective effects may be, first, that the dose of *c*-AUCB employed in HanSD rats was not sufficient to block sEH activity in this normotensive strain. This possibility, however, seems unlikely, because this dose of *c*-AUCB increased the EETs/DHETEs ratio in HanSD, albeit much less than in TGR. In addition, in our previous study we demonstrated that this dose of *c*-AUCB resulted in plasma concentrations of *c*-AUCB that were above the range of the IC_{50} for the specific sEH inhibition and they were observed to inhibit sEH activity

effectively in both *in vitro* and *in vivo* studies [20,21]. Secondly, an alternative and more likely explanation is that the moderate increases in the EETs/DHETEs ratio in response to chronic sEH inhibition in HanSD rats, which exhibit a physiological (normal) availability of biologically active epoxygenase metabolites, is by itself not sufficient to elicit cardioprotective actions. This notion is supported by a recent report of Chaudhary et al. [41], who demonstrated that the addition of exogenous EETs to *c*-AUCB-treated hearts did not further improve the post-ischaemic recovery of cardiac function. Thirdly, it should be particularly noted that the present study, in contrast with previous ones, evaluated the effects of chronic sEH inhibition on myocardial I/R injury. Therefore it is conceivable that acute increases in the availability of EETs can elicit the cardioprotective effect, which is different from that of chronically elevated EETs levels, especially in animals that do not exhibit deficiency of endogenous EETs. This notion is further supported by our finding that acute pretreatment with 14,15-EEZE did not modify the infarct size in normotensive HanSD rats. Nevertheless, additional studies are needed to address this issue.

The fourth important finding of the present study is that chronic treatment with *c*-AUCB did not alter plasma and tissue AngII in TGR and therefore the observed antihypertensive and cardioprotective effects of the sEH inhibitor cannot be ascribed to alterations in the activity of circulating and tissue RAS in this AngII-dependent model of hypertension.

Finally, we demonstrated that untreated TGR and HanSD rats did not reveal any significant differences in tissue concentrations of HETEs, and neither the chronic treatment with sEH inhibitor *c*-AUCB nor acute administration of 14,15-EEZE modified tissue concentrations of HETEs in TGR and HanSD rats. These findings are of special importance because previous studies have shown that either endogenous activation of CYP hydroxylase enzymatic pathway or administration of exogenous 20-HETE had significant detrimental effects on infarct size induced by I/R [42,43]. Our data clearly indicate that chronic pharmacological blockade of sEH as well as acute blockade of EETs receptor(s) did not alter the second major CYP enzymatic pathway and that the cardioprotective effect of the treatment with *c*-AUCB can be ascribed to the enhancement of the concentration of biologically active epoxygenase metabolites.

In summary, our results show that chronic treatment with a novel sEH inhibitor, *c*-AUCB, elicited substantial increases in the availability of endogenous biologically active epoxygenase metabolites and was associated with a marked decrease of BP and limitation of myocardial infarct size induced by I/R in TGR in the phase of established hypertension. Since the cardioprotective action of *c*-AUCB treatment was completely prevented when TGR were pretreated with a selective EETs antagonist, it appears that the cardioprotective effect of sEH inhibition is mediated by EETs actions at the cellular level. Collectively, these findings indicate that chronic inhibition of sEH exhibits antihypertensive and cardioprotective actions in this transgenic model of AngII-dependent hypertension.

AUTHOR CONTRIBUTION

Jan Neckář, Ivan Netuka and Jiří Malý performed the myocardial infarction experiments, Libor Kopkan and Luděk Červenka carried out the BP measurements and AngII determination, and designed the experiments, Zuzana Husková and Herber Kramer performed the determination of the CYP eicosanoids, František Kolář and Bohuslav Oštádal carried out the invasive measurements of cardiac function, František Papoušek performed the echocardiography analysis, Sung Hee Hwang, Bruce Hammock and John Imig synthesized the sEH inhibitor and carried out pharmacokinetic measurements.

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SUPPLEMENTARY ONLINE DATA

Inhibition of soluble epoxide hydrolase by *cis*-4-[4-(3-adamantan-1-ylureido)cyclohexyl-oxy]benzoic acid exhibits antihypertensive and cardioprotective actions in transgenic rats with angiotensin II-dependent hypertension

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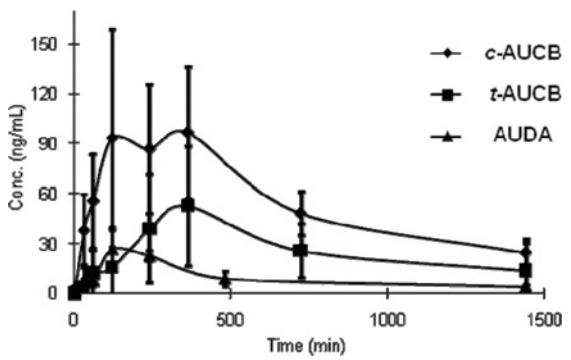

Figure S1 Pharmacokinetic analysis of *c*-AUCB, *t*-AUCB and AUDA with a dose of 5 mg/kg of body weight in rats

AUDA, 12-(3-adamantan-1-ylureido)dodecanoic acid.

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Table S1 Chemical structures, and a comparison of the potencies of *c*-AUCB, *t*-AUCB and AUDA for sEH from various animal species

Data for AUDA and *t*-AUCB are taken from Hwang et al. [1]. AUDA, 12-(3-adamantan-1-ylureido)dodecanoic acid.

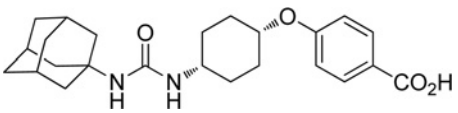
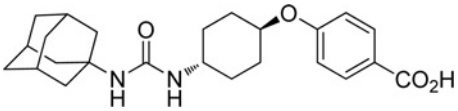
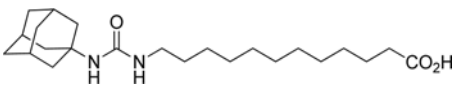
			IC_{50} (nM)		
Name	Chemical structure	Melting point (°C)	Human	Mouse	Rat
c-AUCB		178–187	0.89 ± 0.04	4	7
t-AUCB		250–255	1.3 ± 0.05	8	8
AUDA		142–143	3	10	11

Table S2 The plasma–time profile of *c*-AUCB and *t*-AUCB with the doses of 1 and 5 mg/kg of body weight in rats

PK, pharmacokinetics; AUC, area under the curve. Surprisingly, at a dose of 1 mg/kg of body weight, *t*-AUCB cannot be detected.

	c-AUCB		*t*-AUCB
PK parameter	1 mg/kg of body weight	5 mg/kg of body weight	5 mg/kg of body weight
Half-life (h)	12.8	11.6	6.0
AUC_{0-t} (ng/ml per h)	320	1400	790
AUC_{0-inf} (ng/ml per h)	460	1900	870

REFERENCE

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