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Inhibition of soluble epoxide hydrolase by *cis*-4-[4-(3-adamantan-1-yl-ureido)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 *cis*-4-[4-(3-adamantan-1-yl-ureido)cyclohexyl-oxy]benzoic acid (*c*-AUCB), a novel inhibitor of soluble epoxide hydrolase (sEH), which is responsible for the conversion of biologically active epoxyeicosatrienoic acids (EETs) to biologically inactive dihydroxyeicosatrienoic acids (DHETEs), on blood pressure (BP) and myocardial infarct size in male heterozygous Ren-2 transgenic rats (TGR) with established hypertension. Normotensive Hannover Sprague-Dawley (HanSD) rats served as controls. Myocardial ischemia was induced by coronary artery occlusion. Systolic BP was measured in conscious animals by tail-plethysmography. *c*-AUCB was administered 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/DHETEs – was accompanied by a significant reduction in BP and 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-epoxyeicosa5(Z)-enoic acid), supporting the notion that the improved cardiac ischemic 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.

Keywords

hypertension; angiotensin II; kidney; epoxyeicosatrienoic acids; soluble epoxide hydrolase inhibitor; myocardial ischemia/reperfusion injury

1. Introduction

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

An increasing body of evidence indicates that cytochrome P-450 (CYP)-dependent metabolites of arachidonic acid (AA), such as the epoxyeicosatrienoic acids (EETs), play an important role in the regulation of vascular tone and extracellular fluid volume homeostasis [10-12]. Recent studies have demonstrated that a reduced availability of biologically active EETs as the result of increased conversion of EETs to biologically inactive dihydroxyeicosatrienoic acids (DHETEs), significantly contributes to the pathophysiology of hypertension in the Ren-2 transgenic rat strain (TGR) [13,14]. TGR represents a unique angiotensin II (ANG 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 Hannover Sprague-Dawley (HanSD) 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 soluble epoxide hydrolase (sEH), an enzyme responsible for the conversion of EETs to DHETEs, on blood pressure (BP) and myocardial resistance to I/R have not been evaluated in TGR. We hypothesized that treatment with a sEH inhibitor in this monogenic model of ANG II-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 *cis*-4-[4-(3-adamantan-1-yl-ureido)cyclohexyloxy]benzoic acid (*c*-AUCB) on the BP, myocardial infarct size and the incidence and severity of ischemic and reperfusion arrhythmias in normotensive HanSD 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.

2. Materials and 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.

2.1. 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 (we acknowledge the generous gift of Drs. Bader and Ganten). The TGR rat strain was constructed by inserting the mouse *Ren-2* renin gene, including 5 kb of 5'-flanking

sequences and 9 kb 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 previously described [15]. Animals were fed a standard rat chow containing 0.4 % sodium chloride (SEMED, Prague, Czech Republic) with free access to tap water.

2.2. 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, the crystalline *c*-AUCB (13 mg) was dissolved in ethanol (5 ml) and cyclodextrin (150 mg) after 5 min sonication and this solution was added to the 1 L of water. Hydrogencarbonate (3 ml/L) was given to ensure that the water did not become acidic since low pH can cause the compound 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 to *trans*-isomer, *t*-AUCB. 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 Supplemental material.

The putative selective EETs antagonist, 14,15-epoxyeicosa5(Z)-enoic acid (14,15-EEZE) (Cayman Chemical, MI, USA) 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 based on previous studies that demonstrated that it prevented the cardioprotection induced by either exogenous or endogenous EETs in the canine heart [16].

2.3. Experimental design

2.3.1. 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:

1. TGR + vehicle (n = 5)
2. HanSD + vehicle (n = 5)
3. TGR + *c*-AUCB (n = 6)
4. HanSD + *c*-AUCB (n = 6)

Animals were randomly divided into the aforementioned experimental groups and remained untreated from weaning until 99 days of age. Systolic BP (SBP) was measured every other day in appropriately trained conscious animals by tail-plethysmography via a tail-cuff apparatus (MC 4000; Hatteras Instruments Co., NC, USA) from 88 days of age on. On each occasion SBP was determined as the average of 4 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 the appropriate treatment was started for 48 hours. 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 °C until assay. Urinary concentrations of EETs and DHETEs were measured by ELISA according to the manufacturer's instructions (Detroit R&D Inc., Detroit, MI, USA) using commercially available kits as verified in our previous study [23]. At the end of the experiment rats were sacrificed by overdosing of pentobarbital sodium (Sanofi-Sante Animale, Libourne, France)

and the ratio of left ventricular (LV) weight to tibia length (LVW/TL) was calculated to evaluate the degree of cardiac hypertrophy [21].

2.3.2. 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 hrs prior to initiating the coronary artery occlusion. Studies were performed in the following experimental groups:

1. TGR + vehicle (n = 11)
2. HanSD + vehicle (n = 8)
3. TGR + *c*-AUCB (n = 12)
4. HanSD + *c*-AUCB (n = 10)
5. TGR *c*-AUCB + 14,15-EEZE (n = 9)
6. 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 anesthetized with pentobarbital sodium (60 mg/kg, i.p.). A heparinized cannula was placed in the left carotid artery for BP monitoring with a pressure transducer (Gould P23Gb; Gould Instruments Systems, Valley View, OH, USA) and data subsequently analyzed by our custom-designed software. Rats were ventilated with room air at 68 strokes/min (tidal volume, 1.2 ml/100 g of BW) using a rodent ventilator (Ugo Basile, Italy). Heart rate (HR) was derived from the BP curve. The rectal temperature was maintained between 36.5 and 37.5 °C by a servo-controlled heated table throughout the experiment. A left thoracotomy was performed, and a silk suture 5-0 (Chirmax, Czech Republic) was placed loosely around the LAD coronary artery 1-2 mm distal to its origin. After 10-min stabilization, regional ischemia 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 ischemic tissue continued for 3 hours.

Determination of the infarct size and arrhythmias: Then, hearts were excised and washed with saline through the aorta. The infarct area (IA) and the area at risk (AR) were delineated as described previously [24] by staining with potassium permanganate and 2,3,5-triphenyltetrazolium chloride (Sigma Aldrich, Prague, Czech Republic), 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 LV was determined and the AR was normalized to the LV (AR/LV). 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 ischemic insult and during early reperfusion were assessed according to the Lambeth Conventions [25]. Briefly, premature ventricular complexes (PVCs) 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 (VT) and fibrillation (VF) was also evaluated. The severity of arrhythmias in each group was evaluated by means of a 5-point arrhythmia score as described elsewhere [26].

2.3.3. Series 3: Effects of *c*-AUCB on ANG II concentrations—Animals were divided into the following experimental groups and were exposed to the same treatment protocol as in series 1:

1. TGR + vehicle (n = 8)
2. HanSD + vehicle (n = 7)
3. TGR + *c*-AUCB (n = 8)
4. HanSD + *c*-AUCB (n = 7)

Since it is now recognized that plasma and tissue ANG II concentrations in anesthetized animals are higher than those obtained from decapitated conscious rats and that normotensive animals exhibit greater increases in renin secretion in response to anesthesia and surgery than ANG II-dependent hypertensive intrarenal renin-depleted animals [22], in the present study rats from each experimental group were killed by decapitation and plasma and tissue collected. Plasma, whole kidney, and LV myocardial ANG II levels were measured by radioimmunoassay as described previously [22]. This approach for blood and tissue sampling and ANG II 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 renin-angiotensin system (RAS) in the pathophysiology of hypertension and tissue damage [14,21-23].

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

1. TGR + vehicle (n = 8)
2. HanSD + vehicle (n = 7)
3. TGR + *c*-AUCB (n = 8)
4. HanSD + *c*-AUCB (n = 7)
5. TGR *c*-AUCB + 14,15-EEZE (n = 8)
6. HanSD + *c*-AUCB + 14,15-EEZE (n = 8)

Five minutes after intrajugular acute administration of either 500 μ l of saline vehicle (groups 1 to 4) or 250 μ g of 14,15-EEZE (dissolved in the same volume) rats were euthanized with an excess dose of intravenous pentobarbital sodium and tissues collected. The level of the AA metabolites, EETs, DHETEs and HETEs were measured in the kidney cortex and LV myocardium. 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 [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. Data 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 [34]. Similarly, 5-HETEs; 8-HETEs; 9-HETEs; 11-HETEs, 12-HETEs; 15-HETEs; 19-HETEs and 20-HETEs, respectively, were analyzed 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].

2.3.5. 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

1. TGR + vehicle (n = 9)
2. HanSD + vehicle (n = 9)
3. TGR + *c*-AUCB (n = 9)
4. HanSD + *c*-AUCB (n = 8)

Evaluation of geometrical and functional parameters of the LV was performed using GE Vingmed System Seven (GE Vingmed Ultrasound, Horten, Norway) with 14 MHz linear matrix probe and method established in our laboratory [29,30]. Animals were anesthetized by inhalation of 2% isoflurane. For baseline echocardiographic evaluation, the following diastolic and systolic dimensions of the LV were measured: posterior and anterior wall thickness and cavity diameters. From these dimensions, the main functional parameter, fractional shortening (FS) was derived by the following formula: $FS = (LV \text{ cavity diameter in diastole} - LV \text{ cavity diameter in systole} / LV \text{ cavity diameter in diastole})$ (expressed as a percentage).

After echocardiographic examination, the LV function was assessed invasively using 2F catheter-tip manometer (Millar Instruments, Houston, TX, USA) introduced into the LV cavity *via* the right carotid artery as described earlier [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 heart rate.

2.4. Statistical Analysis

All values are expressed as means $\pm$ SEM. Graph-Pad Prism software (Graph Pad Software, San Diego, CA, USA) was used and 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. 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 Fischer's exact test. Values exceeding the 95% probability limits ($P < 0.05$) were considered statistically significant.

3. Results

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

As shown in Figure 1A, TGR rats before 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, $P < 0.05$ vs. initial values). SBP in HanSD remained within the normotensive range throughout the entire 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 vs. 22.4 ± 0.9 , $P < 0.05$), which was not altered by the treatment with *c*-AUCB.

Figure 1B summarizes the availability of biologically active epoxygenase metabolites when expressed as the ratio of EETs/DHETEs. The basal ratio (before treatment with *c*-AUCB) was significantly lower in TGR than in HanSD rats (1.09 ± 0.15 vs. 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 to 3.28 ± 0.13 , respectively, when compared with basal values ($P < 0.05$ in both strains). However, this increase in the EETs/DHETEs ratio was markedly higher in TGR than in HanSD rats (Figure 1C).

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

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

3.3. Series 3: Effects of α -AUCB treatment on ANG II concentrations

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

3.4. Series 4: Effects of α -AUCB and 14,15-EEZE treatment on EETs, DHETEs and HETEs concentrations

As shown in Figure 4A, untreated TGR revealed significantly lower renal concentrations of EETs than HanSD rats (641 ± 36 vs. 781 ± 34 ng/g of tissue, $P < 0.05$); treatment with α -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 α -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 4C summarizes the results on the intrarenal availability of biologically active epoxigenase metabolites expressed as EETs/DHETEs ratio. This ratio was markedly lower in untreated TGR than in HanSD rats. Treatment with α -AUCB caused significant increases in this ratio in TGR as well in HanSD rats. However, as shown in Figure 4D, this increase in the EETs/DHETEs ratio was markedly higher in TGR than in HanSD rats (215 ± 9 vs. $44 \pm 4\%$, $P < 0.05$). The acute pretreatment with 14,15-EEZE did not alter renal concentrations of EETs and DHETEs in α -AUCB-treated TGR as well as HanSD rats.

As shown in Figure 5A, myocardial levels of EETs in untreated TGR were significantly lower than in HanSD rats (335 ± 22 vs. 401 ± 36 ng/g of tissue, $P < 0.05$). Treatment with α -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 α -AUCB resulted in significant decreases of DHETEs in TGR as well as HanSD rats (Figure 5B). Figure 5C summarizes the results on the availability of biologically

active epoxygenase metabolites in the myocardium when expressed as EETs/DHETEs ratio. This ratio was significantly lower in untreated TGR than in HanSD rats (3.81 ± 0.19 vs. 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 5D, this increase in myocardial EETs/DHETEs ratio was substantially greater in TGR than in HanSD rats (386 ± 21 % vs. 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.

3.5. 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 , heart rate 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 cardiac functional parameters were not modified by the *c*-AUCB treatment.

4. 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/DHETEs. In addition, our results show that chronic inhibition of sEH elicited markedly higher increases in the ratio of EETs/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 to 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 the sodium-hydrogen exchanger [32] and also decrease sodium reabsorption in the cortical collecting duct by blocking the epithelial sodium channels (ENaC) [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/DHETEs – to levels even greater than observed in HanSD rats. Therefore, based on this knowledge and on our current 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 vasodilatation and it has been also demonstrated that EETs oppose the vasoconstrictor action of ANG II [34]. Based on these data 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 ANG II [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 ischemic 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 is 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 end-points 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 HanSD rats, except for systolic pressure and dP/dt. 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 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 ischemic 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 a *trans*-4-[4-(3-adamantan-1-yl-ureido)cyclohexyloxy]benzoic acid (*t*-AUCB) – another newly developed sEH inhibitor – significantly improved the post-ischemic 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 ANG II-dependent model of hypertension results in marked increase in biological availability of EETs and their actions in the myocardium subsequently result in enhanced cardiac ischemic 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 IC₅₀ for the specific sEH inhibition and they were observed to inhibit sEH activity effectively in both *in vitro* and *in vivo* studies [20,21]. Second, 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 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-ischemic recovery of cardiac function. Third, it should be particularly noted that our study in contrast to previous reports 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. 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 ANG II 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 ANG II-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 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 ANG II-dependent hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Changes in systolic blood pressure in TGR (heterozygous Ren-2 renin transgenic rats) either untreated (vehicle) or treated with soluble epoxide hydrolase inhibitor (*c*-AUCB). (B) Urinary excretion ratio of EETs to DHETEs in HanSD (transgene-negative) and TGR rats before and after treatment with *c*-AUCB. (C) Changes in urinary excretion ratio of EETs to DHETEs in HanSD and TGR rats in response to *c*-AUCB administration. Values are mean $\pm$ SEM. * $P < 0.05$ versus corresponding HanSD; $^{\dagger}P < 0.05$ versus corresponding untreated group; $^{\ddagger}P < 0.05$ versus values before treatment.

Figure 2.

(A) Myocardial area at risk (AR) normalized to the size of left ventricle (LV) (AR/LV) in HanSD rats (transgene-negative) and TGR (heterozygous Ren-2 renin transgenic rats) either untreated (vehicle), treated with c-AUCB, or a combination of c-AUCB and a selective EETs antagonist (14,15-EEZE). (B) Infarct size (IA) expressed as percentage of the AR (IA/AR) in untreated and c-AUCB-treated HanSD and TGR rats. Values are mean $\pm$ SEM.

* $P < 0.05$ versus corresponding HanSD; † $P < 0.05$ versus untreated TGR.

Figure 3. Plasma (A), kidney (B) and left ventricular myocardial (C) concentrations of angiotensin II (ANG II) in HanSD rats (transgene-negative) and TGR (heterozygous Ren-2 renin transgenic rats) either untreated (vehicle) or treated with c-AUCB. Values are mean $\pm$ SEM. * P < 0.05 versus corresponding HanSD.

Figure 4. Epoxyeicosatrienoic acids (EETs) (A), dihydroxyeicosatrienoic acids (DHETEs) (B), ratio of EETs to DHETEs (C) and changes in ratio of EETs to DHETEs (D) in kidney cortex of HanSD rats (transgene-negative) and TGR (heterozygous Ren-2 renin transgenic rats) either untreated (vehicle), treated with *c*-AUCB, or a combination of *c*-AUCB and a selective EETs antagonist (14,15-EEZE). Values are mean $\pm$ SEM. * P <0.05 versus corresponding HanSD. [†] P <0.05 versus corresponding untreated group.

Figure 5.

Epoxyeicosatrienoic acids (EETs) (A), dihydroxyeicosatrienoic acids (DHETEs) (B), ratio of EETs to DHETEs (C) and changes in ratio of EETs to DHETEs (D) in left ventricular myocardium of HanSD rats (transgene-negative) and TGR (heterozygous Ren-2 renin transgenic rats) either untreated (vehicle), treated with *c*-AUCB, or a combination of *c*-AUCB and a selective EETs antagonist (14,15-EEZE). * $P < 0.05$ versus corresponding HanSD. Values are mean $\pm$ SEM. † $P < 0.05$ versus corresponding untreated group.

Figure 6.

Concentrations of hydroxyeicosatetraenoic acids (HETEs) in the kidney cortex (A) and in the left ventricular myocardium (B) of HanSD rats (transgene-negative) and TGR (heterozygous Ren-2 renin transgenic rats) either untreated (vehicle), treated with *c*-AUCB, or a combination of *c*-AUCB and a selective EETs antagonist (14,15-EEZE). Values are mean $\pm$ SEM.

Table 1

Left ventricular basal functional parameters evaluated by echocardiography and invasive technique in untreated and *c*-AUCB-treated HanSD and TGR rats.

Parameters	HanSD + vehicle	HanSD + <i>c</i> -AUCB	TGR + vehicle	TGR + <i>c</i> -AUCB
<i>Echocardiography</i>				
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
Fractional shortening (%)	45.2 ± 1.6	43.9 ± 0.5	43.1 ± 1.1	42.7 ± 0.9
<i>Invasive technique</i>				
Peak systolic pressure (mmHg)	120 ± 2	121 ± 5	165 ± 3 [*]	150 ± 5 ^{*†}
+(dP/dt) _{max} (mmHg.sec ⁻¹)	8311 ± 212	8592 ± 229	9993 ± 195 [*]	9146 ± 218 ^{*†}
-(dP/ dt) _{max} (mmHg.sec ⁻¹)	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
Heart rate (beats/min)	342 ± 11	331 ± 7	355 ± 10	326 ± 7

Values are mean ± SEM; +(dP/dt)_{max}, maximum rate of pressure rise; -(dP/ dt)_{max}, maximum rate of pressure fall; τ, time constant of relaxation.

^{*} P<0.05 versus HanSD rats;

[†] P<0.05 versus untreated TGR rats.