Inhibition of soluble epoxide hydrolase counteracts the development of renal dysfunction and progression of congestive heart failure in Ren-2 transgenic hypertensive rats with aorto-caval fistula

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SUMMARY

The detailed mechanisms determining the course of congestive heart failure (CHF) in hypertensive subjects with associated renal dysfunction remain unclear. In Ren-2 transgenic rats (TGR), a model of angiotensin II (ANG II)-dependent hypertension, CHF was induced by volume overload achieved by creation of the aorto-caval fistula (ACF). In these rats we investigated the putative pathophysiological contribution of epoxyeicosatrienoic acids (EETs) and compared it with the role of the renin-angiotensin system (RAS). We found that untreated ACF TGR exhibited marked intrarenal and myocardial deficiency of EETs and impairment of renal function. Chronic treatment of these rats with cis-4-{4-(3-adamantan-1-yl-ureido)cyclohexyl}benzoic acid (c-AUCB, 3 mg/L in drinking water), an inhibitor of soluble epoxide hydrolase (sEH) which normally degrades EETs, increased intrarenal and myocardial EETs, markedly improved survival rate, and increased renal blood flow, glomerular filtration rate and fractional sodium excretion, without altering RAS activity. Chronic angiotensin-converting enzyme inhibition (ACEi) with trandolapril, (6 mg/L in drinking water) improved survival rate even more, and also inhibited the development of renal dysfunction; these beneficial actions were associated with significant suppression of the vasoconstrictor/sodium retaining axis and further activation of the vasodilatory/natriuretic axis of the systemic and intrarenal RAS, without modifying tissue availability of biologically active fatty acid epoxides. In conclusion, these findings strongly suggest that chronic sEH inhibition and chronic treatment with ACEi, each of them altering a different vasoactive system, delay or even prevent the onset of decompensation of CHF in ACF TGR, probably by preventing the development of renal dysfunction.

Key words: aorto-caval fistula, congestive heart failure, epoxyeicosatrienoic acids, hypertension, renal dysfunction, renin-angiotensin system, soluble epoxide hydrolase.

INTRODUCTION

Over the past 30 years substantial progress has been made in the treatment of acute coronary syndromes, however, many surviving patients develop severe myocardial damage which is often followed by congestive heart failure (CHF).1 CHF represents a serious public health problem with an estimated prevalence of 38 million patients worldwide. Unless an authentic progress in the prevention and treatment is achieved, a yearly increase in the number of new patients with CHF is expected to approximate 50%.1–3 In spite of an array of therapeutic approaches available, the prognosis of patients with CHF is poor.1–3 The risk of death increases as a result of cardiac remodelling, impaired renal haemodynamics and sodium retention;4,5 the course of such changes is further accelerated by hypertension.1,4,6 Therefore, exploration of the pathophysiological mechanisms underlying cardiac remodelling and renal dysfunction are needed to improve the prognosis of CHF.

In recent studies of cardiovascular and renal regulations, considerable attention has been focused on the role of epoxyeicosatrienoic acids (EETs), the metabolites of cytochrome P-450 (CYP)-dependent epoxigenase pathway of arachidonic acid (AA) metabolism.7–10 It has been proposed that EETs exhibit antihypertensive actions that are related both to their direct vasodilatory effects and to inhibition of the renal tubular transport of sodium.7,8,10 It has been suggested that intrarenal EETs operate as a compensatory system with protective actions against increased renin-angiotensin system (RAS) activity.11–13 Although direct therapeutic potential of EETs is limited because of their
biological instability, tissue EET concentrations can be increased by blocking soluble epoxide hydrolase (sEH), an enzyme responsible for fast EETs conversion to biologically inactive dihydroyxycicosatrienoic acids (DHETEs). sEH inhibitors prevented and reversed pressure overload- and angiotensin (ANG II)-induced cardiac hypertrophy, reduced infarct size in cardiac ischemia/reperfusion (I/R) and attenuated progression of cardiac remodeling.\textsuperscript{14-17} In an experimental model, an alteration of the gene encoding sEH (Ephx2) facilitated the progression from hypertensive cardiac hypertrophy to CHF, and Ephx2 locus was identified as a CHF susceptibility gene in a rat model of hypertension and CHF.\textsuperscript{18} Moreover, it has recently been shown that chronic sEH inhibition attenuates the progression of chronic kidney disease (CKD) in Ren-2 transgenic rats (TGR) subjected to 5/6 renal mass reduction (5/6 NX).\textsuperscript{19}

Taken together, these findings indicate that sEH inhibition leads to increased tissue EET bioavailability and has important antihypertensive, reno- and cardioprotective effects. Therefore, many studies published to date support the notion that sEH inhibition may represent a promising therapeutic approach for the treatment of CHF. However, no evidence has yet been provided to indicate that chronic sEH inhibition results in prolongation of life in individuals with advanced CHF associated with renal dysfunction. The rat with aorto-caval fistula (ACF) presents a well-defined model of CHF due to volume overload, characterized by activation of the RAS, congestion, cardiac remodelling and impairment of renal function; the model has many features in common with untreated human CHF.\textsuperscript{20-28} In addition, TGR are a unique well-defined monogenic model of ANG II-dependent hypertension with endogenous activation of RAS. Thus, two well-recognized critically important detrimental factors promoting the progression of CHF are combined.\textsuperscript{1,2,4,29-32}

The major aim of the present study is, therefore, to evaluate the effects of chronic treatment with sEH inhibitor (sEHi), cis-4-[4-(3-adamantan-1-yl-ureido) cyclohexyloxy]benzoic acid (c-AUCB), on the morbidity and mortality in male TGR with ACF-induced CHF.

To gain a more detailed insight into the possible role of interactions of active CYP-derived metabolites of AA with RAS in the pathophysiology of CHF-related renal dysfunction, the concentrations of EETs, DHETEs, hydroxyeicosatrienoic acid (HETEs), ANG II and angiotensin-1-7 (ANG 1-7) were determined in normotensive transgene-negative Hannover Sprague-Dawley (HanSD) rats and compared the values with those observed in c-AUCB-treated sham-operated TGR and ACF TGR. In addition, the kidney and heart tissue protein expressions were assessed of CYP2J3 and CYP2C23, two major enzymes responsible for EET formation,\textsuperscript{33} and sEH, the enzyme responsible for degradation of EETs, as well as the protein expression of CYP4A1, the enzyme dominantly responsible for HETE production.

These parameters were first assessed in sham-operated TGR and HanSD rats and in untreated ACF TGR studied 5 weeks after ACF operation in order to obtain detailed knowledge of the degree of RAS activation and CYP-derived metabolites production in the phase of compensated CHF before initiation of treatment regimes. Second, effects of 5-week treatment with sEHi or ACEi on the level of RAS activation and CYP-derived formation of AA metabolites were determined because at this stage (i.e. 10 weeks after induction of ACF) untreated ACF TGR still exhibited a 100% survival rate. However, this was just 1 week before the rats began to die and interest was raised regarding whether the treatment regimes altered the RAS activity and availability of CYP-derived metabolites.

Finally, to further elucidate possible mechanisms underlying beneficial action of sEH inhibition on the course of ACF-induced CHF, effects of 5-week treatment with sEHi or ACEi (i.e. 10 weeks after induction of ACF) on renal function were assessed again by clearance methods in a separate groups of animals; also determined were the cardiac structure and function, using echocardiography and invasive pressure-volume analysis of the left ventricle (LV), respectively.

**RESULTS**

**Series 1: Assessment of RAS and CYP metabolites in the early phase after ACF-induced CHF**

Figure 1a,b shows plasma and kidney ANG II levels observed 5 weeks after induction of ACF or sham-operation. Sham-operated TGR showed significantly higher plasma and kidney ANG II levels as compared with sham-operated HanSD rats. Plasma and kidney ANG II concentrations in ACF TGR were markedly higher than in sham-operated TGR (58 ± 9 vs 24 ± 3 fmol/mL and 276 ± 15 vs 62 ± 3 fmol/g respectively; \( P < 0.05 \) in both cases). There were no significant differences in plasma and kidney ANG 1-7 concentrations between sham-operated HanSD and sham-operated TGR. In contrast, plasma and kidney ANG 1-7 concentrations were significantly higher in ACF TGR than those in sham-operated TGR (29 ± 5 vs 8 ± 4 fmol/mL and 67 ± 8 vs 11 ± 3 fmol/g, respectively; \( P < 0.05 \) in both cases) (Fig. 1d,e).

As shown in Fig. 1c,f, there were no significant differences in the renal and LV myocardial tissue availability of biologically active fatty acid epoxides, expressed as the EETs/DHETEs ratio. In contrast, ACF TGR exhibited a dramatic decrease in the renal and LV myocardial availability of EETs (~75% and ~70%, respectively) as compared with sham-operated TGR. However, there were no significant differences in the renal and LV myocardial tissue availability of biologically active \( \omega \)-hydroxylation metabolites (HETEs) between sham-operated HanSD rats, sham-operated TGR and ACF TGR (data not shown).

As shown in Fig. 2a,b, sEH protein expression in renal cortex and LV myocardial tissue were significantly higher in ACF TGR than in sham-operated HanSD rats or sham-operated TGR (data normalized against \( \beta \)-actin). There were no significant differences in the renal and LV myocardial tissue expression of CYP2J3, CYP2C23 and CYP4A1 proteins between experimental groups (data are not shown).

**Series 2: Effects of sEH and angiotensin-converting enzyme (ACE) inhibition on the survival rate**

All sham-operated HanSD rats survived until the end of the experiment. One sham-operated TGR unexpectedly died 32 weeks after sham-operation, all the others survived until the end of experiment (survival rate 93%). As shown in Fig. 3a, untreated ACF TGR began to die by week 6 (i.e. 11 weeks after
induction of ACF) with final survival rate of 14%. The treatment with either sEH inhibition or ACE inhibitor (ACEi) significantly improved the survival rate, to 41% and 79%, respectively, as compared with untreated ACF TGR \( (P < 0.05 \text{ in both cases}) \). However, in ACF TGR the treatment with ACEi improved survival rate significantly more than did sEH inhibition \( (P < 0.05) \).

Fig. 1 Plasma (a) angiotensin II (ANG II) and (d) angiotensin-1-7 (ANG 1-7) and kidney (b) ANG II and (e) ANG 1-7; (c) kidney and (f) myocardial epoxyeicosatrienoic acids (EETs)/dihydroxyeicosatrienoic acids (DHETEs) ratio in sham-operated transgene negative Hannover Sprague–Dawley (HanSD) and sham-operated heterozygous Ren-2 transgenic (TGR) rats and in untreated TGR with aorto-caval fistula (ACF TGR). *\( P < 0.05 \) compared with sham-operated HanSD. #\( P < 0.05 \) compared to sham-operated TGR.

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Series 3: Effects of 5-week treatment with sEHi or ACEi on EETs, DHETEs, ANG II, ANG 1-7, and HETEs concentrations

As shown in Fig. 3b, there were no significant differences in renal tissue availability of biologically active fatty acid epoxides (when expressed as the EETs/DHETEs ratio) between sham-operated HanSD rats and sham-operated TGR, and neither sEHi nor ACEi treatment altered the ratio in sham-operated TGR. ACF TGR showed a dramatic decrease in the renal availability of epoxygenase metabolites as compared with sham-operated HanSD rats, and sEHi treatment significantly increased EETs/DHETEs ratio in ACF TGR, even to values that were significantly higher than observed in sham-operated HanSD rats. In contrast, the treatment with ACEi did not change EETs/DHETEs ratio in ACF TGR. In all experimental groups LV myocardial availability of biologically active fatty acid epoxides showed the same pattern of changes in response to ACF creation and 5-week treatment period with either sEHi or ACEi as the pattern observed in the renal tissue (data not shown).

As shown in Fig. 4a,b, plasma and kidney ANG II levels were significantly higher in sham-operated TGR than in sham-operated HanSD rats (33 ± 4 vs 17 ± 3 fmol/mL and 69 ± 4 vs 41 ± 4 fmol/g, respectively; \( P < 0.05 \) in both cases). sEHi treatment did not alter plasma or kidney ANG II concentrations in sham-operated TGR but, in contrast to ACEi treatment, significantly reduced plasma and kidney ANG II levels in sham-operated TGR, in the case of renal concentrations down to levels that were significantly lower than observed in sham-operated HanSD rats. Plasma and kidney ANG II levels were in untreated ACF TGR markedly higher than in sham-operated TGR (65 ± 9 vs 33 ± 4 fmol/mL and 253 ± 11 vs 69 ± 4 fmol/g, respectively; \( P < 0.05 \) in both cases). sEHi treatment did not modify plasma
and kidney ANG II concentrations in ACF TGR. In contrast, the treatment with ACEi markedly reduced plasma and kidney ANG II concentrations in ACF TGR to levels observed in sham-operated TGR (Fig. 4a,b).

As shown in Fig. 5a,b, plasma and kidney ANG 1-7 levels did not significantly differ between sham-operated HanSD rats and sham-operated TGR and neither sEHi nor ACEi altered them in sham-operated TGR. ACF TGR exhibited significantly higher plasma and kidney ANG 1-7 than in sham-operated TGR (37 ± 8 vs 9 ± 4 fmol/mL and 54 ± 8 vs 10 ± 3 fmol/g, respectively; P < 0.05 in both cases). sEHi treatment did not alter plasma or kidney ANG 1-7 concentrations in ACF TGR. In contrast, ACEi treatment further increased plasma and kidney ANG 1-7 levels in ACF TGR as compared with untreated ACF TGR (96 ± 12 vs 37 ± 8 fmol/mL and 136 ± 19 vs 54 ± 8 fmol/g, respectively; P < 0.05 in both cases).

Series 4: Effects of 5-week treatment with sEHi or ACEi on renal haemodynamics and excretory function

As shown in Fig. 6a, sham-operated TGR were markedly hypertensive as compared with sham-operated HanSD rats. The treatment with sEHi did not change mean arterial pressure (MAP) in sham-operated TGR. In contrast, ACEi significantly lowered MAP in sham-operated TGR when compared with their untreated counterparts (102 ± 4 vs 147 ± 4 mmHg, P < 0.05). Untreated ACF TGR had lower MAP when compared with sham-operated HanSD rats (101 ± 2 vs 116 ± 3 mmHg, P < 0.05). The treatment with sEHi did not change MAP in ACF TGR. In contrast, the treatment with ACEi significantly lowered MAP in this group when compared with the untreated group (85 ± 2 vs 101 ± 2 mmHg, P < 0.05).

As shown in Fig. 6b,c, untreated ACF TGR exhibited significantly lower glomerular filtration rate (GFR) and renal blood flow (RBF) as compared with sham-operated HanSD rats and with
their untreated or sEHi- or ACEi-treated sham-operated TGR counterparts. The treatment with either sEHi or ACEi significantly increased GFR and RBF in ACF TGR, to values observed in sham-operated HanSD rats.

As shown in Fig. 6d,e, untreated ACF TGR displayed substantially lower urine flow and fractional sodium excretion as compared with sham-operated HanSD rats and with their untreated or sEHi- and ACEi-treated sham-operated TGR counterparts. The
treatment with either sEH or ACEi increased urine flow and fractional sodium excretion in ACF TGR to levels observed in sham-operated HanSD rats.

As shown in Fig. 6e, untreated ACF TGR showed markedly higher fractional potassium excretion than observed in sham-operated HanSD rats and in untreated or sEH- or ACEi-treated sham-operated TGR. The treatment with either sEH or ACEi normalized fractional potassium excretion in ACF TGR to levels found in sham-operated HanSD rats.

Series 5: Effects of 5-week treatment with sEH or ACEi on basal cardiac function assessed by echocardiography and by pressure-volume analyses

Table 1 summarizes the evaluation of cardiac function by echocardiography. Untreated sham-operated TGR exhibited significant cardiac hypertrophy (expressed as heart weight (HW) to body weight (BW) ratio) as compared with untreated sham-operated HanSD rats. The treatment with sEH did not alter the degree of cardiac hypertrophy in sham-operated TGR. In contrast, the treatment with ACEi normalized the HW/BW ratio in sham-operated TGR to values not significantly different from those in sham-operated HanSD rats. Untreated ACF TGR showed a marked increase in HW/BW as compared with untreated sham-operated TGR and the treatment with sEH did not prevent this increase. In contrast, ACEi significantly attenuated the increase in cardiac hypertrophy in ACF TGR. Untreated ACF TGR exhibited increased stroke volume and cardiac output (dependent on the presence of the shunt), significant increases in LV and right ventricle (RV) diameter, and a decrease in the relative LV wall thickness (indicating the development of eccentric hypertrophy), and a significant decrease in LV fractional shortening (indicative of LV systolic dysfunction) as compared with sham-operated TGR and sham-operated HanSD rats. The treatment with sEH did not change any of these parameters in ACF TGR or in sham-operated TGR. In contrast, the treatment with ACEi significantly decreased LV and interventricular septum thickness in sham-operated TGR, which was reflected by the decrease in relative LV wall thickness in these animals. In addition, the treatment with ACEi significantly reduced LV systolic diameter, LV diastolic volume and stroke volume in ACF TGR as compared with untreated ACF TGR.

Table 2 summarizes the assessment of basal cardiac function by left ventricular pressure-volume analysis. Untreated ACF TGR showed significantly lower LV peak pressure than sham-operated TGR. Both sEH and ACEi treatments significantly lowered LV peak pressure in ACF TGR. Untreated ACF TGR exhibited significantly higher LV end-diastolic pressure than untreated sham-operated TGR. The treatment with sEH did not significantly change LV end-diastolic pressure in ACF TGR while ACEi treatment significantly lowered it as compared with untreated ACF TGR. Untreated ACF TGR displayed markedly higher LV end-diastolic volume as compared with sham-operated TGR. The treatment with either sEH or ACEi did not change LV end-diastolic volume in ACF TGR. There were no significant differences in maximum rates of pressure rise (+dP/dt)max between untreated sham-operated TGR and untreated ACF TGR. The treatment with sEH or ACEi did not significantly change +dP/dt)max in ACF TGR. Untreated sham-operated TGR exhibited significantly greater maximum rates of pressure fall (−dP/dt)max as compared with untreated ACF TGR. The treatment with sEH or ACEi did not alter −(dP/dt)max in ACF TGR. Untreated ACF TGR showed markedly lower end-systolic pressure-volume relationship as compared with untreated sham-operated TGR. Untreated ACF TGR also demonstrated lower end-diastolic pressure-volume relationship compared with untreated sham-operated TGR, indicating enhanced LV compliance dependent on chamber eccentric remodelling. The treatment with sEH or ACEi did not change any of those parameters in ACF TGR as well as ACF TGR experimental groups. These representative loops further underscore our notion that untreated ACF TGR exhibit substantial ventricular dilatation as observed from marked increases of volume. Even if indices of

### Table 1 Echocardiography at week 10 after aorto-caval fistula and after 5 weeks pharmacological treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HanSD + water</th>
<th>TGR + water</th>
<th>TGR + sEH</th>
<th>TGR + ACEi</th>
<th>ACF TGR + water</th>
<th>ACF TGR + sEH</th>
<th>ACF TGR + ACEi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight (mg/body weight (g))</td>
<td>3.02 ± 0.10</td>
<td>3.73 ± 0.18</td>
<td>3.68 ± 0.29</td>
<td>2.66 ± 0.16</td>
<td>5.70 ± 0.17*</td>
<td>5.27 ± 0.34*</td>
<td>4.22 ± 0.25*</td>
</tr>
<tr>
<td>RV diastolic diameter (mm)</td>
<td>3.89 ± 0.41</td>
<td>3.36 ± 0.32</td>
<td>3.91 ± 0.31</td>
<td>3.99 ± 0.31</td>
<td>5.49 ± 0.21*</td>
<td>6.19 ± 0.49*</td>
<td>5.26 ± 0.29*</td>
</tr>
<tr>
<td>LV diastolic diameter (mm)</td>
<td>6.39 ± 0.84</td>
<td>6.81 ± 0.41</td>
<td>6.93 ± 0.39</td>
<td>6.81 ± 0.38</td>
<td>10.89 ± 0.25*</td>
<td>10.88 ± 0.48*</td>
<td>9.84 ± 0.36*</td>
</tr>
<tr>
<td>LV systolic diameter (mm)</td>
<td>3.22 ± 0.49</td>
<td>3.75 ± 0.38</td>
<td>3.46 ± 0.38</td>
<td>2.91 ± 0.35</td>
<td>7.09 ± 0.23*</td>
<td>7.07 ± 0.44*</td>
<td>6.19 ± 0.33*</td>
</tr>
<tr>
<td>LV posterior wall thickness in diastole (mm)</td>
<td>2.19 ± 0.31</td>
<td>2.99 ± 0.11</td>
<td>2.33 ± 0.12</td>
<td>2.06 ± 0.09</td>
<td>2.29 ± 0.07</td>
<td>2.39 ± 0.12</td>
<td>2.09 ± 0.08</td>
</tr>
<tr>
<td>Interventricular septum thickness (mm)</td>
<td>2.23 ± 0.19</td>
<td>2.55 ± 0.14</td>
<td>2.37 ± 0.14</td>
<td>2.03 ± 0.09</td>
<td>2.24 ± 0.08</td>
<td>2.54 ± 0.17</td>
<td>2.11 ± 0.12</td>
</tr>
<tr>
<td>LV relative wall thickness (mm)</td>
<td>0.71 ± 0.03</td>
<td>0.86 ± 0.03</td>
<td>0.66 ± 0.03</td>
<td>0.61 ± 0.02</td>
<td>0.41 ± 0.02*</td>
<td>0.43 ± 0.03*</td>
<td>0.42 ± 0.02*</td>
</tr>
<tr>
<td>LV diastolic volume (mL)</td>
<td>0.27 ± 0.09</td>
<td>0.32 ± 0.14</td>
<td>0.33 ± 0.14</td>
<td>0.32 ± 0.13</td>
<td>1.35 ± 0.09*</td>
<td>1.38 ± 0.17*</td>
<td>0.97 ± 0.12*</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>51 ± 3</td>
<td>51 ± 3</td>
<td>50 ± 2</td>
<td>57 ± 4</td>
<td>35 ± 1*</td>
<td>36 ± 3*</td>
<td>37 ± 2*</td>
</tr>
<tr>
<td>Stroke volume (mL)</td>
<td>0.26 ± 0.09</td>
<td>0.27 ± 0.09</td>
<td>0.29 ± 0.10</td>
<td>0.30 ± 0.09</td>
<td>0.96 ± 0.06*</td>
<td>0.98 ± 0.11*</td>
<td>0.73 ± 0.09*</td>
</tr>
<tr>
<td>Cardiac output (mL/min)</td>
<td>112 ± 22</td>
<td>124 ± 31</td>
<td>129 ± 31</td>
<td>139 ± 29</td>
<td>387 ± 19*</td>
<td>389 ± 37*</td>
<td>304 ± 28*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *P < 0.05 compared with sham-operated HanSD.

RV, right ventricle; LV, left ventricle; HanSD, transgene-negative Hannover-Sprague Dawley rats; TGR, Ren-2 renin transgenic rats; ACF, aorto-caval fistula; sEH, soluble epoxide hydrolase inhibitor; ACEi, angiotensin-converting enzyme inhibitor.
Table 2: Invasive hemodynamics at week 10 after aorto-caval fistula and after 5 weeks pharmacological treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>HanSD + water</th>
<th>ACF TGR + water</th>
<th>ACF TGR + ACEi</th>
<th>ACF TGR + sEHi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (b.p.m.)</td>
<td>HanSD + water</td>
<td>406 ± 17</td>
<td>372 ± 18</td>
<td>396 ± 16</td>
<td>364 ± 16</td>
</tr>
<tr>
<td>LV peak pressure (mmHg)</td>
<td>HanSD + water</td>
<td>169 ± 7</td>
<td>131 ± 4</td>
<td>124 ± 0.6</td>
<td>137 ± 4</td>
</tr>
<tr>
<td>LV mean arterial pressure</td>
<td>HanSD + water</td>
<td>140 ± 7</td>
<td>104 ± 5</td>
<td>128 ± 0.6</td>
<td>124 ± 4</td>
</tr>
<tr>
<td>LV end-diastolic pressure</td>
<td>HanSD + water</td>
<td>102 ± 0.7</td>
<td>100 ± 0.7</td>
<td>96 ± 0.7</td>
<td>103 ± 0.7</td>
</tr>
<tr>
<td>LV end-systolic pressure</td>
<td>HanSD + water</td>
<td>111 ± 0.7</td>
<td>100 ± 0.7</td>
<td>110 ± 0.7</td>
<td>120 ± 0.7</td>
</tr>
<tr>
<td>+/(dP/dt)max</td>
<td>HanSD + water</td>
<td>111 ± 0.7</td>
<td>100 ± 0.7</td>
<td>110 ± 0.7</td>
<td>120 ± 0.7</td>
</tr>
<tr>
<td>EDPVRmax (mmHg/mL)</td>
<td>HanSD + water</td>
<td>111 ± 0.7</td>
<td>100 ± 0.7</td>
<td>110 ± 0.7</td>
<td>120 ± 0.7</td>
</tr>
<tr>
<td>LV contractility constant</td>
<td>HanSD + water</td>
<td>111 ± 0.7</td>
<td>100 ± 0.7</td>
<td>110 ± 0.7</td>
<td>120 ± 0.7</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. 
*p < 0.05 compared with sham-operated HanSD (ACF TGR), (b) in sham-operated TGR treated with soluble epoxide hydrolase inhibitor (sEHi), (c) in sham-operated TGR treated with angiotensin-converting enzyme inhibitor (ACEi), (d) in untreated TGR with aorto-caval fistula (ACF TGR), (e) in ACF TGR treated with ACEi, ESPVR, end-systolic pressure volume relationship (blue line); EDPVR, end-diastolic pressure volume relationship (red line).

LV contractility, such as +/(dP/dt)max were not yet significantly altered, these representative loops clearly demonstrate that untreated ACF TGR exhibit marked suppression of the slope of end-systolic pressure volume relationship as compared with sham-operated TGR, which clearly indicates substantial impairment of systolic function in those animals. In addition, these representative loops show that neither sEHi nor ACEi treatment altered any of the cardiac parameters measured. This issue is further elucidated in the Discussion section.

Series 6: Acute effects of ACF creation on blood pressure (BP) and heart rate (HR)

To the best of the authors’ knowledge this is the first study of the effects of ACF creation in TGR, an ANG II-dependent model of hypertension. Therefore, the preliminary phase of this study tested and compared the results with those in sham-operated HanSD rats. As shown in Fig. 8, after an initial profound drop in highly elevated BP after ACF creation (BP fell significantly below that observed in sham-operated HanSD rats), within 56 h the TGRs’ BP returned to values observed in sham-operated HanSD rats. These values of mean
suitable to be employed in the authors’ experiments, the conclusion was reached that the model of ACF is sham-operated HanSD rats. Overall, based on the results of these evaluation of the progression of CHF.

cates that ACF-induced CHF in this ANG II-dependent model concentrations as compared with sham-operated TGR. This indicated transgene negative Hannover Sprague–Dawley (HanSD) rats. All these bene-

cially active fatty acid epoxides substantially contribute to the pro-

gression of CHF in ACF TGR. In contrast, our results regarding CYP-dependent epoxygenase pathway of AA metabolism in the early phase of ACF-induced CHF support our original hypothe-

sis that reduced tissue availability of biologically active fatty acid epoxides, which results from sEH-mediated increased conversion of EETs to DHETEs, plays an important role in the pathophysiology of progression of CHF in ACF TGR.

The second important set of findings is that chronic sEH inhibition with c-AUCB substantially increased the survival rate and prevented the development of renal dysfunction in ACF TGR animals. All these beneficial actions of chronic c-AUCB treatment were associated with normalization of intrarenal and cardiac availability of biologically active fatty acid epoxides. Notably, c-AUCB-treated ACF TGR showed an even higher intrarenal and myocardial EETs/DHETEs ratio than observed in sham-operated animals. The results presented here show that chronic treatment of ACF TGR with sEHi did not significantly change circulating or intrarenal ANG II and ANG 1-7 concentrations, indicating that the beneficial actions of chronic sEH inhibition are not related to alterations in vasoconstrictor or vasodilator axes of the RAS. These findings support the notion that tissue deficiencies of biologically active fatty acid epoxides substantially contribute to the pathophysiology of ACF-induced CHF in TGR and, in particular, to the development of renal dysfunction in this model.

Chronic treatment of ACF TGR with ACEi dramatically improved survival rate and abolished the impairment of renal hemodynamics and excretory function. In this case, these protective

arterial BP are within the range of renal autoregulatory capacity and therefore are adequate for normal baseline renal function in ACF TGR. In addition, as shown in Fig. 8, TGR responded to creation of ACF by a marked compensatory increase in HR which thereafter (within 36 h) returned to the initial level. In the end, ACF TGR exhibited significantly lower heart rate than observed in sham-operated HanSD rats. Overall, based on the results of these experiments, the conclusion was reached that the model of ACF is suitable to be employed in the authors’ chronic studies aimed at evaluation of the progression of CHF.

**DISCUSSION**

The first important set of findings of the present study is that, when assessed 5 weeks after model creation, the ACF TGR showed increased plasma and kidney ANG II and ANG 1-7 concentrations as compared with sham-operated TGR. This indicates that ACF-induced CHF in this ANG II-dependent model of hypertension resulted in further activation of systemic and intrarenal vasoconstrictor/sodium retaining axis of the RAS and it was associated with concomitant marked activation of circulating and renal vasodilator/natriuretic axis of the RAS. The results presented here are in agreement with recent findings of Cohen-Segev et al. who demonstrated that renal and cardiac immunoreactivity of angiotensin-converting enzyme (ACE), the major enzyme responsible for ANG II formation, and angiotensin-converting enzyme type 2 (ACE2), the most important ANG 1-7-forming enzyme, are increased when determined 2 weeks after induction of ACF. In addition, our results show that ACF TGR have substantially reduced intrarenal and cardiac availability of biologically active fatty acid epoxides. Since the renal and cardiac generation of EETs was evidently normal, as indicated by unaltered tissue protein expression of CYP2J3 and CYP2C23, it is likely that the deficient availability was the result of increased conversion of EETs to DHETEs; this is indicated by increased tissue sEH protein expression in these animals. On the other hand, the present results show that intrarenal and myocardial activity of CYP-450-dependent ω-hydroxylase pathway was not significantly changed in ACF TGR as compared with sham-operated TGR and HanSD rats, as indicated by unaltered protein expression of CYP4A1 and HETEs concentrations. These findings are of major importance since it has been suggested that increased activity of CYP-450-dependent ω-hydroxylase pathway of AA metabolism with subsequent increased production of HETEs (mainly 20-hydroxyeicosatrienoic acid (20-HETE)) promote the development of hypertension-associated end-organ damage, pathological cardiac hypertrophy and CHF. Our findings do not confirm this view, indicating that alterations of the CYP-450-dependent ω-hydroxylase pathway do not importantly contribute to the progression of CHF in ACF TGR. In contrast, our results regarding CYP-dependent epoxygenase pathway of AA metabolism in the early phase of ACF-induced CHF support our original hypothesis that reduced tissue availability of biologically active fatty acid epoxides, which results from sEH-mediated increased conversion of EETs to DHETEs, plays an important role in the pathophysiology of progression of CHF in ACF TGR.

The second important set of findings is that chronic sEH inhibition with c-AUCB substantially increased the survival rate and prevented the development of renal dysfunction in ACF TGR animals. All these beneficial actions of chronic c-AUCB treatment were associated with normalization of intrarenal and cardiac availability of biologically active fatty acid epoxides. Notably, c-AUCB-treated ACF TGR showed an even higher intrarenal and myocardial EETs/DHETEs ratio than observed in sham-operated animals. The results presented here show that chronic treatment of ACF TGR with sEHi did not significantly change circulating or intrarenal ANG II and ANG 1-7 concentrations, indicating that the beneficial actions of chronic sEH inhibition are not related to alterations in vasoconstrictor or vasodilator axes of the RAS. These findings support the notion that tissue deficiencies of biologically active fatty acid epoxides substantially contribute to the pathophysiology of ACF-induced CHF in TGR and, in particular, to the development of renal dysfunction in this model.

Chronic treatment of ACF TGR with ACEi dramatically improved survival rate and abolished the impairment of renal hemodynamics and excretory function. In this case, these protective

Fig. 8 Time course of (a) mean arterial pressure and (b) heart rate (measured by radiotelemetry) in heterozygous Ren-2 transgenic rats (TGR) before and after induction of aorto-caval fistula (ACF) and in sham-operated transgene negative Hannover Sprague–Dawley (HanSD) rats. *P < 0.05 compared with initial values, i.e. before ACF induction.
actions were not associated with any significant changes in the kidney or myocardial EETs bioavailability but were accompanied by marked suppression of plasma and kidney ANG II levels and further augmentation of circulating and intrarenal ANG 1-7 concentrations. These findings strongly suggest that protective actions of chronic ACEi treatment against the progression of CHF are not related to modification of CYP-dependent epoxygenase pathway. More likely, the suppression of the vasoconstrictor/sodium retaining axis of the RAS in the circulation and kidney tissue combined with a further increase in the activity of the vasodilator/natriuretic axis of the RAS are the main mechanisms of protective actions of ACEi treatment against CHF-related mortality and development of renal dysfunction in ACF TGR.

The third important set of findings relates to the changes in cardiac function. Creation in TGR of ACF resulted after 10 weeks in the development of marked eccentric chamber remodelling and cardiac hypertrophy related to enhanced cardiac output, largely dependent on blood recirculation through the fistula. Although load-dependent measures of LV contractility, such as +(dP/dt)\text{max}, were in this early stage not significantly impaired, the significantly suppressed slope of end-systolic pressure volume relationship and decreased LV fractional shortening indicated marked systolic dysfunction. In this early stage of CHF, untreated ACF TGR also displayed prolonged LV relaxation along with significantly decreased end-diastolic pressure volume relationship. This indicated impairment of early diastolic function with a simultaneous enhancement of end-diastolic chamber compliance. Taken together, these findings indicate that 10 weeks after creation of ACF the untreated ACF TGR are still at the stage of compensated CHF as described inings indicate that 10 weeks after creation of ACF the untreated ACF TGR are still at the stage of compensated CHF as described in previous studies with this volume overload model. Soon afterwards the animals showed progression toward decompensated hypertrophy and heart failure. In this connection, of special interest are the findings regarding effects of chronic treatment regimes on cardiac function. It was found that chronic sEH treatment did not improve cardiac morphology, performance or contractility in ACF TGR, despite the fact that it markedly improved survival rate. Similarly, chronic treatment with ACEi only slightly reduced LV end-diastolic pressure and, consequently, the stroke volume. These findings suggest that renal impairment rather than left ventricular dysfunction is an important determinant of long term survival in this particular CHF model. Otherwise, the other cardiac function characteristics assessed by echocardiography or pressure-volume analyses were not significantly altered as compared with untreated ACF TGR; nevertheless, a substantial improvement of survival rate was observed.

In view of beneficial effects of chronic sEH treatment on the course of CHF in ACF TGR, it is noteworthy to mention that in our recent study, it was found that chronic sEH inhibition in normotensive ACF HanSD rats did not improve the survival rate in CHF and did not attenuate the development of renal dysfunction. Markedly, chronic treatment with ACEi considerably improved the survival rate and inhibited the development of renal dysfunction in ACF HanSD rats. The recognition of the different effects of sEH inhibition on the course of CHF in TGR and HanSD rats underscores the importance of the interaction of hypertension, RAS and CYP-derived metabolites in the progression of CHF-related mortality and development of renal dysfunction.

Collectively, the present results show that chronic sEH inhibition with c-AUCB normalized tissue availability of EETs, significantly improved survival, and prevented the development of renal dysfunction in ACF TGR without altering RAS activity. Chronic treatment with ACEi exhibited even more pronounced beneficial effect on the survival rate, and also inhibited the development of renal dysfunction. These beneficial actions were associated with significant suppression of the vasoconstrictor/sodium retaining axis and further activation of vasodilatory/natriuretic axis of the systemic and intrarenal RAS, without modifying tissue availability of biologically active fatty acid epoxides. In conclusion, these findings strongly suggest that in ACF TGR chronic sEH inhibition and chronic ACEi treatment, each of them altering a different vasoactive system, delay or even prevent the onset of decompensation in CHF. It is proposed that these protecting effects are achieved by predominantly renal mechanisms.

**METHODS**

**Ethical approval and animals**

The studies were performed 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 #16-2012 issued by Ministry of Health of Czech Republic), which accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All animals used in the present study were bred at the Centre of Experimental Medicine of this Institute, from stock animals supplied by the Max Delbrück Centre for Molecular Medicine, Berlin, Germany, which is accredited by the Czech Association for Accreditation of Laboratory Animal Care. Heterozygous TGR were generated by breeding male homozygous HanSD rats and female homozygous HanSD rats and age-matched HanSD rats served as transgene-negative normotensive controls. The animals were kept on a 1200 hour/1200 hour light/dark cycle. Throughout the experiments rats were fed a normal salt, normal protein diet (0.45% NaCl, 19–21% protein) manufactured by SEMED (Prague, Czech Republic) and had free access to tap water.

**CHF model and chronic treatments**

Congestive heart failure was induced by volume overload which results from induction of ACF, using a needle technique (18-gauge needle, diameter 1.2 mm) as originally described by Garcia and Diebold, and employed and validated by many investigators including our own group. Sham-operated rats underwent a similar procedure but without creating ACF. In accordance with the recommendation for BP measurement in experimental animals, we employed a radiotelemetry system for direct BP measurements. TGR and HanSD rat (aged 9 weeks) were anaesthetized with a combination of tiletamine, zolazepam (8 mg/kg, Zoletil; Virbac SA, Carros Cedex, France), and xylazine (4 mg/kg, Rometa; Spofa, Prague, Czech Republic) intramuscularly, and TAl11PA-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 48 h and then ACF was created in TGR (n = 4) and, after a 6-h period needed to recover from acute surgery, BP and heart rate were recorded over 96 h. Sham-operated HanSD rats (n = 4) served as controls. The results from this pilot study are shown...
in series 6. The sEHi used, c-AUCB, was prepared freshly and given in drinking water at 3 mg/L. The appropriate amount of c-AUCB was dissolved with gentle warming in polyethylene glycol and added with rapid stirring to warm drinking water to obtain a 0.1% aqueous solution of polyethylene glycol. The dose of c-AUCB was selected based on recent studies where it elicited substantial increases in tissue concentration of EETs without altering RAS activity. The c-AUCB dose was chosen that blocks sEH activity without altering plasma and tissue ANG II levels with an intention to separate and assess the effect of EETs elevation alone on the course of ACF-induced CHF. Since therapeutic regimes involving inhibition of RAS are common or even standard in the therapy of CHF, the treatment with sEHi was also employed to compare the effects with those obtained in the c-AUCB-treated groups. Trandolapril (6 mg/L, in drinking water, Gopten, Abbot, Prague, Czech Republic), was used to inhibit ACE because in previous studies and here in the preliminary experiments it was demonstrated that at this dose the drug provided maximal blockade of RAS and was well tolerated both by rats with ACF-induced CHF and by sham-operated animals.

Series 1: Assessment of RAS and CYP metabolites in the early phase after ACF-induced CHF

The aim here was to evaluate the degree of activation of the two axes of the RAS: the vasoconstrictor ACE/ANG II axis, and the vasodilator ACE2/ANG 1-7 axis, together with determination of the rate of synthesis along the two CYP-dependent pathways, those of epoxygenase and o-hydroxylase. Rats at the initial age of 9 weeks were divided into the following experimental groups and the follow-up period was 5 weeks:

1. Sham-operated HanSD rats + vehicle (water) treated (n = 8)
2. Sham-operated TGR + water treatment (n = 8)
3. ACF TGR + water treatment (n = 11)

Since it is now well recognized that ANG II and ANG 1-7 concentrations in anaesthetized animals are higher than those measured in decapitated conscious rats, and that normotensive animals exhibit a greater increase in renin secretion in response to anaesthesia and surgery than observed for ANG II-induced hypertension in renal-depleted animals at the end of experiments plasma and tissue samples were obtained without anaesthesia. Plasma and whole-kidney ANG II concentrations were assessed by radioimmunoassay (RIA) based on the original procedure developed by Fox et al. and further modified and validated in our laboratory. This procedure is described in detail in our previous studies. Plasma and kidney ANG 1-7 levels were also measured by RIA as validated and in detail described in our recent studies.

This approach enabled the comparison of the present results with those from earlier studies by the authors of the role of the RAS in the pathophysiology of various cardiovascular diseases. The levels of EETs and DHETEs in the kidney cortex and LV tissue were measured. The samples were extracted, the extracts were separated by reverse-phase high performance liquid chromatography and analysed by negative-mode electrospray ionization and tandem mass spectroscopy as described previously. Specifically, 8,9-EETs; 11,12-EETs and 14,15-EETs were measured separately and then pooled and presented jointly. These metabolites are the most active products formed in the CYP epoxygenase pathway. The EETs/DHETEs ratio was calculated from total concentrations of EETs and of DHETEs. Western blot analysis of protein expression of CYP2C23 and CYP23, the enzymes that are predominantly responsible for the formation of EETs, and of sEH, the enzyme responsible for the conversion of EETs to DHETEs, were performed as described previously, with levels normalized against β-actin. In addition, HETEs concentrations and protein expression for CYP4A1, the enzyme responsible for the formation of HETEs, were analysed in the renal and LV tissues as described previously.

Series 2: Effects of sEH or ACE inhibition on the survival rate

Male rats of the same age as in series 1 (9 weeks) derived from several litters were randomly assigned to experimental groups, to make sure that animals from a single litter did not prevail in any group. Animals underwent either sham-operation of ACF creation as described above (on the week labelled as 0) and were left without treatment for 5 weeks. Previous studies have shown that 5–10 weeks after ACF operation cardiac remodelling and renal functional characteristics typical for CHF become apparent. Since we have found in our preliminary experiments that the development of CHF characteristics in TGR is accelerated as compared with normotensive animals, we intentionally shortened the period without treatment to 5 weeks, because at that time TGR are at the stage of compensated CHF and still exhibit 100% survival. At this time point (week 0) the rats were divided in the following experimental groups:

1. Sham-operated HanSD rats + water (initial n = 12)
2. Sham-operated TGR + water (initial n = 14)
3. ACF TGR + water (initial n = 54)
4. ACF TGR + sEHi (initial n = 52)
5. ACF TGR + ACEi (initial n = 44)

The follow-up period was 50 weeks. The rats were inspected daily and body weight (BW) was determined three times per week. In addition, always the same experienced technician (PS) monitored animals for the presence of CHF symptoms using a scoring system that was developed and verified previously. Briefly, each animal was scored with respect to five features of rat CHF: (i) presence of raised fur (piloerection); (ii) diminished activity (lethargy); (iii) peripheral cyanosis; (iv) rapid or laboured breathing (dyspnea); and (v) abdominal swelling (ascites). Each symptom was scored on the scale from 0 to 3 points and a total CHF score was calculated for each animal as a sum of individual points.

Series 3: Effects of 5-week treatment with sEHi or ACEi on EETs, DHETEs, ANG II, ANG 1-7, and HETEs concentrations

Animals were prepared as described in Series 2 and on week 0 the pharmacological treatment was initiated for a period of 5 weeks. At the end of experiments (on week +5) the rats were killed by decapitation and plasma and kidney ANG II, ANG 1-7, EETs, DHETEs and HETEs were measured as described in Series 1. The following experimental groups were examined:

1. Sham-operated HanSD rats + water (n = 7)
2. Sham-operated TGR + water (n = 7)
3. Sham-operated TGR + sEHi (n = 7)
4. Sham-operated TGR + ACEi (n = 7)
Series 4: Effects of 5-week treatment with sEHi or ACEi on renal haemodynamics and excretory function

The following experimental groups exposed to the same protocol as animals in Series 3 were evaluated:

1. Sham-operated HanSD rats + water (n = 8)
2. Sham-operated TGR + water (n = 8)
3. Sham-operated TGR + sEHi (n = 7)
4. Sham-operated TGR + ACEi (n = 7)
5. ACF TGR + water (n = 8)
6. ACF TGR + sEHi (n = 9)
7. ACF TGR + ACEi (n = 9)

At the end of the experimental protocol (on week 6), the rats were anaesthetized and acute clearance experiments were performed as described in detail in our previous studies, to determine renal haemodynamics and excretory parameters.40,47,49

Series 5: Effects of 5-week treatment with sEHi or ACEi on basal cardiac function assessed by echocardiography and by pressure-volume analysis

In this series the following groups, subjected to the same protocol as in Series 3, were studied:

1. Sham-operated HanSD rats + water (n = 6)
2. Sham-operated TGR + water (n = 6)
3. Sham-operated TGR + sEHi (n = 7)
4. Sham-operated TGR + ACEi (n = 7)
5. ACF TGR + water (n = 9)
6. ACF TGR + sEHi (n = 9)
7. ACF TGR + ACEi (n = 9)

Statistical analysis

Statistical analysis of the data was performed using Graph-Pad Prism software (Graph Pad Software, San Diego, CA, USA). Analysis of variance (ANOVA) for repeated measurements, followed by Student-Newman–Keuls test, was performed for analysis within groups (e.g. survival rate). Statistical comparison of other results was made by the Student’s t-test, Wilcoxon’s signed-rank test for unpaired data or one-way ANOVA when appropriate. Unless otherwise indicated, values are expressed as mean ± SEM. A P-value < 0.05 was considered statistically significant.

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