Cardiac Endothelin System Impairs Left Ventricular Function in Renin-Dependent Hypertension via Decreased Sarcoplasmic Reticulum Ca\textsuperscript{2+} Uptake

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**Background**—We evaluated the role of the cardiac endothelin (ET) system in compensated hypertensive left ventricular (LV) hypertrophy (LVH) and after the transition toward LV dysfunction.

**Methods and Results**—Hypertensive transgenic rats overexpressing the Ren2 gene (Ren2 rats) were investigated between the ages of 10 and 30 weeks (Ren2-10 and Ren2-30 groups, respectively) and compared with age-matched normotensive Sprague-Dawley (SD) rats (SD-10 and SD-30 groups, respectively). Systolic blood pressure and LV weight were elevated in both Ren2 groups compared with their age-matched SD control groups (P < 0.0001). In Ren2-30 rats, LV end-diastolic pressure increased and −dP/dt\textsubscript{max} decreased compared with the values in SD-30 and Ren2-10 rats (P < 0.05). This was paralleled by an activation of LV mRNA expression of preproET-1 and ET-converting enzyme-1 and ET subtype A (ETA) receptor binding in Ren2-30 compared with Ren2-10 rats (P < 0.001). Cardiac fibrosis was increased and sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} reuptake was reduced in Ren2-30 compared with SD-30 and Ren2-10 rats (P < 0.05). Treatment of Ren2 rats with the selective ETA receptor antagonist Lu135252 between 10 and 30 weeks of age did not lower systolic blood pressure, heart weight, or cardiac fibrosis but completely prevented the deterioration of LV end-diastolic pressure and abolished alterations in −dP/dt\textsubscript{max} and SR Ca\textsuperscript{2+} reuptake compared with no treatment in Ren2-30 and SD-30 rats (P < 0.05).

**Conclusions**—Activation of the cardiac ET system accounts at least in part for the LV dysfunction that gradually develops in LVH. The protective effect of ETA antagonism can be attributed to the improvement of diastolic LV function that is due to normalization of impaired SR Ca\textsuperscript{2+} uptake. (*Circulation*. 2000;102:1582-1588.)

**Key Words:** hypertrophy ■ hypertension ■ endothelin ■ renin ■ sarcoplasmic reticulum

It remains enigmatic why an initially adaptive response like left ventricular (LV) hypertrophy (LVH) eventually deteriorates to LV dysfunction and finally even to overt failure. The endothelin (ET) system may play an important role in this transition. ET-1 is a potent vasoconstrictor peptide that is considered to be of great importance in cardiac physiology and pathophysiology. ET-1 exerts its effects through stimulation of 2 functionally different receptors, ET subtype A (ETA) and ET subtype B (ETB), which are widely distributed in the cardiovascular system. Local ET-1 production in the heart has been reported, with secretion from the endocardium, myocardium, and coronary endothelium\textsuperscript{1,2} and from cardiac fibroblasts.\textsuperscript{3} ET-1 is generated by the conversion of 2 precursors, preproET-1 and proET-1. In the heart, this conversion is controlled by ET-converting enzyme (ECE), the membrane-bound isoform of which is named ECE-1. An early hallmark in the transition from hypertensive LVH to LV failure is the development of diastolic LV dysfunction. The latter may be caused by different alterations in the myocyte and/or nonmyocyte compartment of the heart. In particular, impairment of sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} handling and excessive collagen matrix buildup could both occur. ET-1 can alter SR Ca\textsuperscript{2+} handling and can induce matrix genes that mediate cardiac fibrosis.\textsuperscript{4} Whether the ET system is activated during the transition from LVH to LV dysfunction and is of pathophysiological significance to any of the postulated mechanisms during this transition is unknown.

The transgenic hypertensive TGR(mRen2)27 rat model provides a well-established model of renin-dependent arterial hypertension and cardiac hypertrophy.\textsuperscript{5} The introduction of the mouse Ren2 gene (mRen2) led to a genetic model of renin-dependent hypertension, in which increased levels of cardiac...
angiotensin II have been described. As a result, an important part of the cardiac pathophysiology has been ascribed to activation of the cardiac renin-angiotensin system. We used this model to address the hypothesis that the cardiac ET system is activated during and contributes to the transition from compensated to decompensated LVH.

Methods

All animal experiments were conducted in accordance with institutional guidelines and the German Animal Protection Law.

Experiment 1

In the first experiment, we determined the activity of the ET system in the early and late stage of LVH in untreated TGR(mRen2)27 (Ren2 rats) compared with age-matched Sprague-Dawley (SD) rats. The early stage of LVH was investigated at the age of 10 weeks (Ren2-10 and SD-10 groups), and late stage of LVH was studied at the age of 30 weeks (Ren2-30 and SD-30 groups).

Experiment 2

To further explore the potential role of the cardiac ET system, we assessed the effect of selective ETA blockade on LV hypertrophy and function. For this aim, Ren2 rats were orally treated between 10 and 30 weeks of age with 30 mg·kg⁻¹·d⁻¹ of Lu135252 (Knoll AG) in standard rat chow (SSNIF GmbH).

Animals

Male heterozygous Ren2 and SD rats were purchased from M&B (Bomholtvej, Denmark) at the age of 8 weeks. All rats were randomly assigned to be investigated at the age of 10 weeks (Ren2-10 and SD-10, n=8 each), to be killed at the age of 30 weeks (Ren2-30 and SD-30, n=20 and n=10, respectively), or to be treated for 20 weeks with Lu135252 (Ren2-30/Lu135252, n=20). At the age of 10 or 30 weeks, the rats were subjected to LV catheterization (see below) under pentobarbital anesthesia. Thereafter, EDTA blood samples were taken. The heart was rapidly excised, rinsed in a 0.9% NaCl solution, and blotted dry. The right ventricle and LV were separated and weighed, and a transverse slice of the LV was immersed in Dubosq-Brasil solution (aqueous solution of 53% ethanol, 12% formaldehyde, 7% acetic acid, and 0.5% picric acid; all compounds from Merck KG) for histological studies. The remaining ventricular tissue was frozen in liquid nitrogen and stored at -80°C until further analysis.

Blood Pressure Measurement

Systolic blood pressure (SBP) was determined in awake rats by use of a tail cuff and pressure transducer in conjunction with a computerized pressure delivery and chart recording system (TSE Biosystems GmbH).

Determination of LV Function

Each rat was anesthetized with an intraperitoneal injection of pentobarbital (60 mg·kg⁻¹). The right carotid artery was isolated and cannulated with a solid-state pressure transducer catheter (Micro-Tip 3F, Millar Instruments), which was connected to a personal computer (TSE Biosystems GmbH). The catheter was advanced into the LV for measurement of pulsatile LV pressure (LVP), maximal LVP (LVPmax), and LV end-diastolic pressure (LVEDP). As indices of global contractility and relaxation, we determined the maximal rates of increase and decrease in LVP (±dP/dt) and −dP/dt, respectively.

Histological Evaluation

All samples were embedded in paraffin, cut into 3-μm sections, and subjected to hematoxylin-eosin and Sirius red staining as a specific dye for connective structures. Cardiac fibrosis was quantified after Sirius red staining by morphometry with use of a video camera combined with a video control system (Sony MC-3255, AVT-Horn GmbH) adapted to a Zeiss Axiophot microscope. Image analysis was performed with the use of freely available software (Scion Image 1.62a, Scion Co) on a Power Macintosh 8200/120 computer. After digitalization, gray-scale images were transformed into binary images, and the relation of Sirius red-stained area (connective tissue) to total area of the heart section was determined.

Molecular and Biochemical Analysis

Plasma ET-1 enzyme immunoassay, binding assay for ETA and ETB receptor, and Northern blot analysis were carried out as previously reported.

Determination of SR Ca²⁺ Reuptake

Tissue homogenates were prepared at 4°C from a 50-mg specimen of frozen LV tissue under stringent phosphoprotein protection conditions. Protein was determined by the Lowry method. Homogenate supernatants were used for determination of SR Ca²⁺ uptake, which was measured at 37°C by using a vacuum filtration technique as described. The medium contained (mmol/L) imidazole 40 (pH 7.0), KCl 100, MgCl₂ 5, Tris-ATP 5, phosphocreatine 6, potassium oxalate 10, NaF 10, ETGTA 0.2, and CaCl₂ 0.1 (15 dpm/mmol) corresponding to 0.21 μmol/L free Ca²⁺, with 3 mg wet tissue wt/mL. Termination of the reaction after 2 minutes, filtration, and determination of radioactivity associated with dried 0.45-μm Millipore HAWP filters were described earlier. The reaction was linear for at least 4 minutes and allowed for Ca²⁺ uptake into SR vesicles only, with ATP-dependent Ca²⁺ transport into mitochondrial vesicles being inhibited by NaN₃. Thapsigargin and the ionophore A23187 at 10 μmol/L prevented Ca²⁺ uptake completely. The rate of Ca²⁺ uptake (expressed in nmol Ca²⁺·min⁻¹·mg protein⁻¹) is defined as the rate of oxalate-supported Ca²⁺ uptake related per milligram of whole tissue protein.

Statistical Evaluation

All data are expressed as mean±SEM. Statistical analysis was performed by 2-tailed Student t test and 2-way ANOVA followed by the Bonferroni adjustment. Analysis of correlation was performed by the Pearson coefficient. Differences were considered significant at a level of P<0.05.

Results

Experiment 1

Arterial Pressure and LV Weight

Development of SBP in heterozygous TGR(mRen2)27 is shown in Figure 1. SBP was significantly elevated in Ren2 compared with SD rats (P<0.0001). Data for LV weight and body weight are presented in Table 1. Normalized LV weight (LV wt · body wt⁻¹) was higher in Ren2-10 (+38%, P<0.0001) and Ren2-30 (+32%, P<0.0001) rats compared with age-matched SD control rats.

LV Function

The data for LV function are presented in Figure 2. Significant deterioration of LV function was observed in the Ren2 rats. LVEDP, which was not elevated in the beginning of the
experiment in young Ren2-10 compared with SD-10 rats, increased 43% in old Ren2-30 compared with Ren2-10 rats and was 87% higher in Ren2-30 compared with SD-30 control rats at the end of the observation period. This was paralleled by a further significant deterioration of diastolic $dP/dt$ ($dP/dt/LVP_{max}$) in Ren2 rats at 30 weeks of age (Ren2-30 versus Ren2-10, $P<0.05$). Systolic $dP/dt$ ($1\ dP/dt/LVP_{max}$) did not change in Ren2 rats during the experiment. In contrast to the Ren2 rats, age had a significant effect on both $1\ dP/dt/LVP_{max}$ (SD-30 versus SD-10, $P<0.05$) and $2\ dP/dt/LVP_{max}$ (SD-30 versus SD-10, $P<0.05$) in SD rats. However, unlike in Ren2 rats, LVEDP was maintained at a normal level in the SD control rats.

**ETA and ETB Receptor Binding**

The data for LV ET receptor binding are presented in Table 2. ETA receptor binding was detected in both strains, but the density of cardiac ETA receptors increased markedly in the Ren2-30 rats compared with control rats. LV ETA receptor density was 35% lower in Ren2-10 rats compared with SD-10 rats. This was reversed in Ren2-30 rats, in which there was a 27% elevated ETA receptor density compared with that in SD-30 rats. ANOVA indicated a significant interaction between strain and age for ETA receptor density ($P<0.0001$). ETA receptor density was 24% higher in SD-30 compared with SD-10 rats, whereas the elevation in Ren2-30 compared with Ren2-10 rats was significantly higher ($+142\%$). ETA receptor affinity was significantly increased in the Ren2-30 compared with SD-30 rats. No specific LV ETB receptor binding was detected for any groups.

**ET-1 Plasma Levels**

Plasma ET-1 levels are presented in Table 2. In general, plasma ET-1 increased in Ren2-30 rats, whereas it remained stable in SD rats. Plasma ET-1 concentrations were not altered in Ren2-10 compared with SD-10 rats. In contrast, plasma ET-1 concentration was elevated by 78% in Ren2-30 compared with SD-30 control rats. Plasma ET-1 levels did not increase with age in SD rats, but these levels were 138% higher in Ren2-30 compared with Ren2-10 rats.

**Expression of ET-1, ECE-1, and Atrial Natriuretic Factor mRNA**

Data for LV mRNA levels are summarized in Figure 3. Representative Northern blots are shown in Figure 3d. LV expression analysis for preproET-1 did not reveal any significant differences between the strains. In both strains, the expression of LV preproET-1 increased with age ($P<0.0001$). PreproET-1 was 2.3-fold higher in SD-30 compared with SD-10 rats and 2.5-fold higher in Ren2-30 compared with Ren2-10 rats (Figure 3a).
LV ECE-1 expression did not differ in Ren2-10 compared with SD-10 rats but was 40% elevated in Ren2-30 compared with SD-30 rats. Age significantly affected ECE-1 expression in both strains (\(P<0.0001\)). ECE-1 mRNA levels were 42% higher in SD-30 compared with SD-10 rats and 82% higher in Ren2-30 compared with Ren2-10 rats (Figure 3b).

LV atrial natriuretic factor (ANF) expression was 24.5-fold higher in Ren2-10 compared with SD-10 rats and 14-fold higher in Ren2-30 compared with SD-30 rats. Age had a significant effect on ANF expression in both strains (\(P<0.05\) vs SD-30; \#\(P<0.05\) vs Ren2-10). ANF mRNA levels were 5.5-fold elevated in SD-30 compared with SD-10 rats and 3.1-fold higher in Ren2-30 compared with Ren2-10 rats (Figure 3c).

Cardiac Fibrosis

Data for LV fibrosis are shown in Figure 4. Two representative histological slices with Sirius red staining are presented in Figure 4c. Perivascular fibrosis was 2.2-fold elevated in Ren2-10 compared with SD-10 rats and 4.6-fold higher in Ren2-30 compared with SD-30 rats. No increase in perivascular fibrosis was seen in the aged SD rats. In contrast, in the older Ren2 rats, perivascular fibrosis increased 1.9-fold (Figure 4a). There was no significant interstitial fibrosis in the younger Ren2 rats compared with age-matched SD rats. In contrast, there was a clear interstitial fibrosis with a 4.7-fold higher score in the older Ren2 rats compared with age-matched SD-30 rats. Interstitial fibrosis did not change in aging SD rats but increased 2.6-fold in Ren2-30 compared with Ren2-10 rats (Figure 4b).
Rate of Ca\textsuperscript{2+} Reuptake by the SR

Data for SR Ca\textsuperscript{2+} uptake are shown in Figure 5. SR Ca\textsuperscript{2+} uptake was higher in Ren2-10 rats compared with SD-10 rats, but this did not reach statistical significance ($P=0.13$). SR Ca\textsuperscript{2+} uptake was significantly reduced by 29% in Ren2-30 compared with SD-30 control rats. Age had a no effect on Ca\textsuperscript{2+} uptake in SD rats, but Ca\textsuperscript{2+} uptake was 40% lower in Ren2-30 compared with Ren2-10 rats.

Experiment 2

Treatment with the selective ETA receptor antagonist Lu135252 completely prevented the increase in LVEDP and the decrease of $-\frac{dP}{dt}$/LVP\textsubscript{max} in the Ren2-30/Lu135252 rats compared with the untreated Ren2-30 rats (Figure 2a and 2c). This effect was independent from SBP, because Lu135252 did not lower SBP (Figure 1) and had no effect on LV weight in the Ren2-30/Lu135252 rats compared with untreated Ren2-30 rats (Table 1). SBP was even somewhat higher at the age of 28 weeks in the Ren2/Lu135252 compared with untreated Ren2 rats ($\Delta +20$ mm Hg, $P=0.007$; Figure 1). In the Ren2-30/Lu135252 rats, no alteration in cardiac perivascular or interstitial fibrosis was found (Figure 4). In contrast, Lu135252 significantly improved SR Ca\textsuperscript{2+} reuptake in the Ren2-30/Lu135252 rats compared with the untreated Ren2-30 rats (Figure 5). Furthermore, a highly significant correlation between diastolic function and SR Ca\textsuperscript{2+} reuptake was found in the groups of SD-30, Ren2-30, and Ren2-30/ Lu135252 rats ($r=0.80$, $P<0.001$), whereas no correlation was found between systolic ventricular function and SR Ca\textsuperscript{2+} uptake in these groups.

Discussion

The current set of experiments was designed to investigate whether an activated ET system contributes to the progressive loss of LV function in a rat model of renin-dependent LVH.

Development of Progressive LV Dysfunction in Ren2 Rats

The present study is the first to present data on progressive cardiac dysfunction in aged Ren2 rats. LV catheterization revealed an increased LVEDP and reduced diastolic function in 30-week-old rats compared with 10-week-old rats. These findings indicate that the transition from compensated LVH in 10-week-old Ren2 (Ren2-10) rats is predominantly attributable to diastolic impairment in 30-week-old Ren2 (Ren2-30) rats. The deterioration of cardiac function was paralleled by a strong increase of LV ANF expression, indicating the shift from compensatory LVH in young Ren2-10 rats to a dysfunctional type of LV hypertrophy in old Ren2-30 rats.

Role of Myocyte and Nonmyocyte Compartment in Progression of LV Dysfunction

Increased collagen content has previously been implicated to be responsible for increased wall stiffness in LV dysfunction.\textsuperscript{12} An altered composition of the extracellular matrix has also been reported in the Ren2 model.\textsuperscript{5,13}

Our investigation using histological analysis with Sirius red staining revealed no alteration in interstitial fibrosis but an increased perivascular fibrosis in young Ren2-10 rats compared with SD-10 control rats. This finding is in agreement with previous observations in 16-week-old Ren2 rats.\textsuperscript{13} Furthermore, both perivascular and interstitial fibrosis were elevated in Ren2-30 rats. We conclude that our results reflect the dynamic changes in interstitial matrix composition that were due to aging in the Ren2 model.

To examine whether reduced LV function can be attributed to altered properties of the cardiomyocyte compartment, we determined SR Ca\textsuperscript{2+} transport, which is known to be closely related to cardiomyocyte function.\textsuperscript{14} The SR Ca\textsuperscript{2+} reuptake rate was not reduced in the compensated state of LVH but strikingly decreased in the older dysfunctional Ren2 rats. Moreover, $-\frac{dP}{dt}$/LVP\textsubscript{max} was strongly and significantly correlated to the activity of SR Ca\textsuperscript{2+} reuptake rate in Ren2-30, SD-30, and Ren-30/Lu135252 rats ($r=0.80$, $P<0.001$). In contrast, systolic ventricular function was not directly correlated to SR function in the Ren2-30 rats. These data suggest that development of dysfunction in the older Ren2 rats resulted from both increased LV fibrosis and impaired SR Ca\textsuperscript{2+} pump function.

Activity of ET System in Compensated LVH

Plasma ET-1 concentration and LV expression of preproET-1 mRNA were not altered in Ren2-10 rats compared with SD-10 control rats. This finding is in agreement with a study showing no change in serum and myocardial ET-1 levels in compensated LVH due to myocardial infarction, in which ETB receptor density was very low.\textsuperscript{17} LV ECE-1 mRNA expression was not altered in the Ren2-10 group. In summary, the compensated state of LVH in the Ren2-10 rats was characterized by a reduced density of LV ETA receptors. The lack of specific ETB receptor binding that we observed in both Ren2 and SD rats is in accordance with data obtained in SD rats with chronic LV dysfunction due to myocardial infarction, in which ETB receptor density was very low.\textsuperscript{17} LV ECE-1 mRNA expression was not altered in the Ren2-10 group. In summary, the compensated state of LVH in Ren2 rats is characterized by unchanged plasma ET-1 concentrations and by unaltered LV preproET-1 and ECE-1 mRNA expression. ETA receptor density is reduced in the compensated state of LVH in Ren2 rats. These findings
suggest that the cardiac ET system in Ren2 rats is normal or even reduced during the compensated state of LVH.

**Activation of Cardiac ET System Parallels Transition to LV Dysfunction**

LV dysfunction occurred in the Ren2-30 rats and was accompanied by elevated plasma ET-1 levels. Several studies have reported an increase in plasma ET-1 concentrations in experimental animal models15,18 and in patients with chronic LV dysfunction.19 It has been demonstrated that plasma ET-1 levels are a major predictor of mortality in patients with chronic LV dysfunction.20 An interesting study revealed that in isolated perfused rat hearts, ET-1 increased LVEDP only in hearts with established LVH.9 Another important argument in favor of an instrumental role for the ET system in the progression of LV dysfunction is that ET blockade improved cardiac function and survival after experimental myocardial infarction.21

Old SD-30 rats had an increased level of ECE-1 mRNA compared with the level in young SD-10 rats, indicating an age-dependent rise in the activity of the ET system in SD rats. In contrast to the preproET-1 expression, LVEDP of older Ren2 rats compared with age-matched control rats. Similar results were reported for rats with LVH after aortic banding, in which cardiac ECE-1 mRNA expression increased only in the very late stage of hypertrophy.9

A striking finding was the upregulation of ETA receptor binding sites in dysfunctional LVH in the Ren2-30 rats. Moreover, ETA receptor affinity was also increased in the old Ren2-30 rats. An increase of ETA receptor density was also reported for heart failure in cardiomyopathic hamsters,22 chronic heart failure due to myocardial infarction in rats,17 and end-stage heart failure due to dilated cardiomyopathy in humans.23 Thus, the transition from compensated LVH in hypertensive Ren2-10 rats to mainly diastolic dysfunction in older Ren2 rats is paralleled by an activation of all components of the cardiac ET system.

**Does an Activated ET System Deteriorate LV Function?**

To assess the functional role of the activated ET system, we chronically treated the Ren2 rats with the selective ETA receptor blocker Lu135252 in the time window during which manifestation of LV dysfunction occurred in these animals. The treatment clearly prevented the development of LV dysfunction in Ren2 rats. This effect was independent of an antihypertensive effect of the compound, because blood pressure was even somewhat increased at the end of the study period during ETA blockade.

One mechanism by which an activated ET system induces LV dysfunction may relate to its stimulatory effect on cardiac fibrosis. Interestingly, however, selective ETA blockade did not change perivascular or interstitial fibrosis in the Ren2-30/Lu135252 group despite a marked activation of the cardiac ET system in untreated Ren2-30 rats. This finding is consistent with a recent study in which selective ETA receptor blockade did not reduce cardiac fibrosis in the 2-kidney, 1-clip model of LVH.8

The second mechanism that may relate to LV dysfunction in renin-dependent LVH is the decreased SR Ca2+ handling that we observed in Ren2-30 rats. A relation between the activity of the ET system and myocyte Ca2+ handling is supported by the finding that SR Ca2+ reuptake was negatively correlated with all components of the ET system in Ren2 rats (preproET-1, $r = -0.64$; ECE-1, $r = -0.71$; ETA receptor density, $r = -0.71$; $P < 0.05$). The strongest evidence for a causal interaction between the ET system and cardiomyocyte Ca2+ handling is our observation that ETA receptor blockade did completely normalize SR Ca2+ handling in the Ren2-30/Lu135252 group. Moreover, the significant correlation between $-dP/dt_{max}$ and SR Ca2+ reuptake rate clearly demonstrates the direct interaction of the ET system and diastolic LV dysfunction in this model. Although the conclusion of an impact of improved SR Ca2+ handling for the observed beneficial effect of the ETA antagonist appears to be reasonable, we cannot rule out fundamental effects on other components of cellular Ca2+ handling, such as sarcoplasmic Na+-Ca2+ exchange or various Ca2+ channels. Moreover, modulatory influences on cellular Ca2+ regulation could arise from alterations of other receptors and signaling pathways that are involved in the control of single components of cellular Ca2+ handling.24 Taken together, the activity of the cardiac ET system was normal or even reduced during the compensated state of LVH in Ren2 rats. The onset of LV dysfunction was characterized by a strong activation of the ET system, which was paralleled by increased cardiac fibrosis and decreased SR Ca2+ transport. Selective ETA receptor antagonism completely prevented the development of LV dysfunction in the Ren2 rats by improving SR Ca2+ handling without affecting blood pressure, LV weight, or cardiac fibrosis. We conclude that the cardiac ET system plays an important role in the transition from LVH to LV dysfunction via impaired SR Ca2+ trafficking in renin-dependent LVH.

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