Reduction in Left Ventricular Messenger RNA for Transforming Growth Factor \( \beta_1 \) Attenuates Left Ventricular Fibrosis and Improves Survival Without Lowering Blood Pressure in the Hypertensive TGR(mRen2)27 Rat

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Abstract—Angiotensin II recruits transforming growth factor \( \beta_1 \) (TGF\( \beta_1 \)) and is related to left ventricular fibrosis. However, it is unclear whether chronic in vivo reduction in left ventricular TGF\( \beta_1 \) expression blunts fibrosis and improves outcome in angiotensin II–dependent hypertension. Four-week-old male hypertensive TGR(mRen2)27 (Ren2) rats received either normal food, low-dose losartan (0.5 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \)), or tranilast (a nonspecific TGF\( \beta \) inhibitor; 400 mg \( \cdot \) kg\(^{-1} \) \( \cdot \) d\(^{-1} \)) \((n=10 \text{ for each group})\) for 12 weeks and were compared with Sprague-Dawley control rats. The effect of tranilast on survival was evaluated in 34 additional untreated homozygous Ren2 rats. Tranilast or low-dose losartan did not lower blood pressure. However, the increase in left ventricular weight (Ren2 versus SD 3.1 \( \pm \) 0.16 versus 2.1 \( \pm \) 0.06 mg/g body wt; \( P<0.05 \)) was significantly \((P<0.05)\) blunted by both tranilast (2.7 \( \pm \) 0.05) and losartan (2.7 \( \pm \) 0.07).

Both drugs prevented the increase in left ventricular TGF\( \beta_1 \) mRNA and fibronectin mRNA and blunted the increase in hydroxyproline content and the increase in perivascular fibrosis. The perivascular fibrosis score correlated significantly with the level of expression of TGF\( \beta_1 \) \((r=0.62; \ P=0.019)\). In situ hybridization demonstrated increases in TGF\( \beta_1 \) mRNA, predominantly in perivascular and nonmyocyte areas. Both drugs did not prevent the decrease in systolic or diastolic dP/dt, but tranilast significantly improved the survival of untreated Ren2 rats \((P=0.029)\). In conclusion, TGF\( \beta_1 \) mRNA expression is increased predominantly in nonmyocyte regions in the hypertrophied left ventricle in this angiotensin II–dependent model of hypertension. This increase is probably due to high angiotensin II levels rather than to hypertension. This is the first study to suggest that chronic inhibition of TGF\( \beta_1 \) expression attenuates left ventricular hypertrophy and fibrosis, even without lowering blood pressure. \((Hypertension. \ 2000;36:747-754.)\)

Key Words: hypertension, experimental hypertrophy, transforming growth factors, fibrosis

Hypertension-related left ventricular hypertrophy is associated with an adverse outcome, although hypertrophy is a normal adaptation to increased loading and is invariably found in every rat model of hypertension. This suggests that only part of the hypertrophic process is maladaptive. It is thought that this maladaptive part of cardiac hypertrophy is related to increased expression of growth factors in the heart, which eventually lead to excess fibrosis. Consequently, it has been proposed that a reduction in growth factors could prevent such adverse changes. A central role in this process has been proposed for transforming growth factor \( \beta_1 \) (TGF\( \beta_1 \)). Although all 3 isoforms of TGF\( \beta \) (TGF\( \beta_1 \), \( \beta_2 \), and \( \beta_3 \)) are present in the heart, the level of the type 1 isoform seems to be particularly related to the development of left ventricular hypertrophy. TGF\( \beta_1 \) is secreted by most cell types and has complex actions, depending on the cell type that is involved: it inhibits the proliferation of many cells but also stimulates the growth of mesenchymal cells and stimulates the formation of extracellular matrix. The level of TGF\( \beta_1 \) mRNA is increased in left ventricular hypertrophy. In spontaneously hypertensive rats (SHR) with heart failure, this increase in left ventricular TGF\( \beta_1 \) expression, accompanied by increased fibronectin expression, has been suggested to mark the transition from stable hypertrophy to failure. In that study, the expression of TGF\( \beta_1 \) was not increased in nonfailing SHR. However, an increased expression of TGF\( \beta_1 \) has also been described in nonfailing hypertrophy as occurs after abdominal aortic coarctation. This suggests that the particular type of hypertension may determine the relative importance of TGF\( \beta_1 \) expression in the development of hypertrophy and
fibrosis. It has been demonstrated in vitro that angiotensins II induces TGFβ in smooth muscle cells and in cardiac cells and fibroblasts, which is underlined by the finding that TGFβ expression is also increased in the hypertrophied left ventricle of the TGR(mRen2)27 (Ren2) rat, a hypertensive model characterized by increased cardiac angiotensin II. Interestingly, the report by Villarruel et al demonstrated only increased perivascular fibrosis and no diffuse fibrosis in this model. Angiotensin II infusion also increases the expression of TGFβ, although this could not be dissected from a direct pressor effect.

Although increased TGFβ has been associated with its profibrotic actions, there is also evidence that TGFβ can have protective, beneficial effects in human atherosclerotic disease. As a result, it is yet unclear whether beneficial or detrimental effects should be expected from an in vivo reduction in the expression of TGFβ in hypertensive heart disease. Therefore, we evaluated the cardiac effects of a reduction in TGFβ mRNA with tranilast in a rat model of hypertension. Tranilast, a drug that was originally used for the treatment of allergic and dermatological diseases, was recently reported to inhibit TGFβ-mediated collagen formation. This compound is now gaining interest in human cardiovascular disease as a possible therapy for restenosis.

The hypothesis we sought to test is that in the hypertensive Ren2 rat, the increased angiotensin II level, independent of its hypertensive effect, augments left ventricular TGFβ expression and thereby increases collagen content, which leads to impaired left ventricular performance and decreased survival. Therefore, we also assessed the effects of tranilast in direct comparison with a nonhypotensive dose of losartan, a specific angiotensin II type 1 (AT1) receptor antagonist. To assess the capacity of either drug to prevent left ventricular changes, treatment was initiated before hypertension was established.

**Methods**

**Drug Treatment**

All rats were purchased from the Moellegaard Breeding Company. Directly after being weaned at 4 weeks of age, 30 male Ren2 rats were randomly assigned to receive no treatment, losartan (0.5 mg · kg⁻¹ · d⁻¹), or tranilast (400 mg · kg⁻¹ · d⁻¹) (n=10 for each group). Tranilast was a generous gift from Dr T. Hama (Kissei Laboratories, Nagano, Japan). The dosage of tranilast was based on previous studies that had shown that the same dosage in Sprague-Dawley (SD) rats significantly decreased the hydroxyproline content in granulation tissue. This dosage is expected to result in serum levels of ~100 μmol/L, which is comparable to the serum concentration in human clinical trials. Because we did not anticipate an effect of the blood pressure, the dosage of losartan was chosen to affect blood pressure as well. The drugs were mixed with the rat chow. We measured at the respective pressure of the rats, these measurements serve only to provide an indication of whether treatment affected medial thickness. Sections were photographed, and these photographs were coded so that the investigator who took the measurements (Y.M.P.) was unaware of the origin of the photograph. Thickness was measured at 4 locations in the carotid artery, all separated by 45°, in 3 photographs of each artery.

**Molecular Studies**

Total RNA was extracted from the left ventricle with TRIzol (GIBCO) reagent, according to the instructions of the manufacturer. Total RNA (15 μg) was denatured with formamide/formaldehyde, size separated with gel electrophoresis, transferred to a nylon membrane (Hybond N; Amersham), and fixed to the membrane through UV cross-linking. The membranes were hybridized with a 32P-labeled 825-bp BamHI fragment of the atrial natriuretic factor (ANF) cDNA cloned in pGEM-4Z (kindly provided by Dr Sigrid Hoffmann, Ruprecht-Karls University Heidelberg, Heidelberg, Germany), a 445-bp polymerase chain reaction–generated cDNA probe for TGFβ1 (primers available on request), and an 800-bp fragment of the fibroactin cDNA (kindly provided by Dr Kenneth Boheler, Gerontology Research Center, Baltimore, Md).

The membranes were hybridized in Quickhyb (Stratagene) solution for 1 hour at 68°C. Thereafter, the membranes were washed in a mixture of 0.1% SDS and decreasing concentrations of standard saline citrate (SSC) at a final temperature of 55°C. Then, the bands were visualized through exposure to x-ray film (Kodak, Germany) for 24 to 48 hours and quantified through digital scanning with the aid of the publicly available NIH Image program. The density of the bands was compared with that of the bands obtained through subsequent hybridization to a GAPDH probe. Results were displayed as the ratio between the density of the target band, relative to the GAPDH band (in arbitrary units).

**Hydroxyproline Measurement**

Hydroxyproline content of the left ventricle was determined according to a previously described method. In brief, a sample of the left ventricle (>100 mg) was hydrolyzed in HCl, after which Ehrlich’s reagent was added. The resultant extinction was compared with a standard curve for the determination of hydroxyproline content.

**Histological Analysis**

The transverse midsection of the left ventricle was immersed in formaldehyde and embedded in paraffin, sections were cut and stained with hematoxylin-eosin, and separate sections were stained with Sirius Red to stain for collagen. The left carotid artery was similarly treated but only stained with hematoxylin-eosin.

Sections were photographed, and these photographs were coded so that the investigator who made the measurements (Y.M.P.) was unaware of the origin of the photograph.

To analyze whether treatment affected myocyte thickness, the thickness of individual myocytes was measured on 3 different spots.
To analyze the changes in fibrotic areas, we digitized the Sirius Red-stained sections and measured the proportion of stained collagen within a predefined, fixed square. This was done directly adjacent to an artery for the measurement of perivascular fibrosis. It was also done in an area covered by myocytes to measure interstitial fibrosis.

Thickness of the carotid artery was measured at 4 locations in the carotid artery, all separated by 45°, in 3 photographs of each artery.

### In Situ Hybridization

Cryosections of left ventricular samples were incubated for 15 minutes in 0.2N HCl and treated with 20 μg/mL Proteinase K for 3 minutes. Then, they were refixed in 4% paraformaldehyde/PBS for 20 minutes and acetylated with 25 mmol/L acetic anhydride in 100 mmol/L triethanolamine for 10 minutes. After being washed twice in PBS, sections were dehydrated with ethanol. Air-dried sections were hybridized under coverslips with 4*10^8 cpm 33P-labeled RNA probe/mL hybridization mixture (50% formamide, 3 mol/L NaH2 PO4, 10% dextran sulfate, 1 mol/L NaCl, 20 mmol/L Tris, 5 mmol/L EDTA, 10 mmol/L NaHPO4, 10% dextran sulfate, 1× Denhardt’s solution, 0.5 mg/mL yeast total RNA) for 20 hours at 55°C in a humidified chamber. After hybridization, coverslips were removed in 5× SSC. The sections were then washed at 55°C in 2× SSC; incubated at 37°C in a buffer composed of 0.5 mol/L NaCl, 10 mmol/L Tris, and 5 mmol/L EDTA containing RNase A (10 μg/mL) for 30 minutes; and washed for 30 minutes at 55°C, followed by dehybridization through ethanol containing 0.3 mol/L ammonium acetate. After drying, the sections were dipped in radiographic emulsion (Amersham), exposed for 2 to 3 weeks at 4°C, and developed according to the manufacturer’s instructions. Counterstaining was performed with hematoxylin-eosin.

### RNA Probe for TGFβ1

A 338-bp portion of the 3′ portion of the murine TGFβ1 gene was amplified from total mouse embryonic cDNA and cloned into a modified pBluescript SK vector. The construct was linearized with appropriate restriction enzymes, and RNA probes were transcribed in the presence of 3P-UTP with T7 polymerase to prepare the antisense probe and T3 polymerase to generate the sense probes. All sections were hybridized with both the antisense and the sense probe.

### Survival Trial

Because tranilast treatment appeared to decrease mortality rates in the heterozygous Ren2 rats, in a separate experiment we assessed the effects of tranilast on survival of untreated homozygous Ren2 rats. If untreated with an ACE inhibitor, the homozygous rats are known to have a very high mortality rate after 8 to 12 weeks.24 Thirty-four untreated with an ACE inhibitor, the homozygous Ren2 rats were randomized to receive either tranilast (400 mg z 2 kg z 2 ) or no treatment. Treatment was started at the age of 4 weeks (immediately after weaning) and continued for 10 weeks.

### Statistical Analysis

All results are shown as mean±SEM. Differences between groups were tested by a 1-way ANOVA, corrected where appropriate by Duncan’s multiple range test for multiple comparisons (all with SPSS for Windows 7.5). The effect of tranilast on survival was tested in the Kaplan-Meier survival analysis with a log-rank test. Correlations were tested by a Pearson correlation test.

### Results

#### Treatment Trial

Neither losartan nor tranilast affected the expected increase in blood pressure, so blood pressure reached similar hypertensive levels in all 3 Ren2 groups (Figure 1). In the untreated Ren2 rat group, 3 rats died, whereas in each of the losartan- and tranilast-treated groups, 1 rat died.

Both systolic and diastolic dp/dt values were significantly decreased in untreated Ren2 rats compared with SD rats.
LVEDP was not increased. Neither losartan nor tranilast attenuated the decrease in systolic or diastolic dP/dt. Heart rate and body weights were similar among all groups.

Left ventricular weight was significantly increased in the untreated hypertensive Ren2 rats, and both losartan and tranilast significantly attenuated this increase in left ventricular weight (Figure 2). Tranilast did not significantly affect left ventricular function parameters or left ventricular weight in normal SD rats.

Aortic Dose-Response to Angiotensin II
The dose-response curve to angiotensin II was not significantly altered in aortic rings from untreated hypertensive Ren2 rats compared with untreated SD rats. This increase was not affected by either treatment. Tranilast did not affect the dose-response curve to angiotensin II, but the nonhypotensive dose of losartan significantly decreased the maximal response to angiotensin II. Tranilast indicates Ren2 rats treated with tranilast; Ren2LOS, Ren2 rats treated with losartan.

Histological Analysis
Myocyte thickness was significantly increased in the untreated Ren2 rats, but this was not affected by either treatment (Figure 4, top). The measurement of fibrosis revealed that perivascular fibrosis was significantly increased in the untreated Ren2 rats (Figure 5, bottom), whereas interstitial fibrosis was unaltered (data not shown). Both losartan and tranilast (Figure 5, bottom) blunted the increase in perivascular fibrosis. The degree of perivascular fibrosis correlated significantly with the level
of expression of TGFβ \((r=0.62; P=0.019)\) and correlated weakly and nonsignificantly with left ventricular weight–to–body weight ratio \((r=0.46; P=0.08)\). Furthermore, in situ hybridization showed that the increased expression in the left ventricle of the untreated Ren2 rat was located mainly in areas that were not occupied by myocytes, such as the perivascular fibrotic areas, and, to a lesser extent, between myocytes in interstitial areas (Figure 6).

The thickness of the media of the carotid artery was significantly increased in the untreated hypertensive Ren2 rats. Both losartan and tranilast failed to affect media thickness (Figure 4, bottom).

**Molecular Studies**

Expression of TGFβ \(_1\) mRNA was significantly increased in left ventricular tissue from untreated hypertensive Ren2 rats. This increase was prevented by both losartan and tranilast and to a similar extent (Figure 7A, left). This was also reflected by similar changes in the expression of fibronectin (Figure 7A, right). The expression of ANF was significantly increased in untreated hypertensive Ren2 rats, which was significantly blunted (but not normalized) by both tranilast and losartan (Figure 7B).

**Hydroxyproline Content**

Total left ventricular hydroxyproline content was increased in untreated Ren2 rats versus SD rats. Both tranilast and losartan blunted this increase (Figure 5, top), so hydroxyproline was not significantly increased in the groups treated with tranilast or losartan.

**Effects of Tranilast on Survival**

The Kaplan-Meier curves in Figure 8 demonstrate the effect of tranilast compared with untreated Ren2 rats. Overall, survival was higher than anticipated for the homozygous rats, because these have been reported to not survive at all without ACE inhibitor treatment. We found a mortality rate of only 41% after 3 months in the untreated homozygous rats. Nevertheless, overall survival was significantly improved in the tranilast treated rats compared with the untreated Ren2 rats \((P=0.029)\).

**Discussion**

In the present study, we tested the hypothesis that increased angiotensin II levels in the hypertensive Ren2 model directly augment left ventricular TGFβ\(_1\) expression and thereby increase collagen content, which leads to impaired left ventricular performance and decreased survival. We postulated that this adverse cascade might be initiated by angiotensin II independent of the increased blood pressure. To address this hypothesis, we compared tranilast, a nonspecific inhibitor of TGFβ expression,\(^{17,18}\) with the effects of a nonhypotensive dose of the angiotensin II type 1 (AT\(_1\)) receptor antagonist losartan.
Our study is the first to report that tranilast reduces left ventricular TGFβ₁ expression, collagen accumulation and perivascular fibrosis, and left ventricular hypertrophy and improves survival in a blood pressure–independent manner. A nonhypotensive dose of losartan similarly blunted the expression of left ventricular TGFβ₁ and had similar effects on collagen and perivascular fibrosis. Interestingly, neither tranilast nor losartan altered myocyte thickness, nor did they improve left ventricular function, suggesting a specific role for TGFβ₁ in nonmyocyte processes. This notion is underlined by our finding that the increase in mRNA for left ventricular TGFβ₁ seems in large part confined to areas outside the myocytes, where we also noted fibrosis. There are only a few studies that have reported the localization of cardiac mRNA for TGFβ₁. Li et al.⁵ showed increased TGFβ₁ mRNA and protein in samples obtained from patients with hypertrophic cardiomyopathy or aortic stenosis. However, the authors of this study also noted an increase in TGFβ₁ in these human cardiac myocytes, which we did not observe in our rat model. In 2 very different rat models of hypertension, the mRNA for TGFβ₁ was initially found only in cultured nonmyocyte cardiac cells, but in this study, the induction of TGFβ₁ was noted in the cardiomyocytes cultured from hypertrophied left ventricles.²⁶ The paucity of reports on the localization of cardiac TGFβ₁ mRNA expression and the different models in which it was investigated do not allow us to draw definite conclusions on the comparability of our findings with those of others. However, the current data do suggest that the increase in TGFβ₁ mRNA colocalizes with cardiac fibrosis.

Taken together, our findings suggest that in the hypertensive Ren2 rat, increased angiotensin II levels augment the expression of left ventricular TGFβ₁ in noncardiomyocytes and that this augments perivascular fibrosis. This is in line with recent data from a separate study that also suggested that the effects of angiotensin II on cardiac fibroblasts might be mediated in large part by TGFβ₁.²⁷ It does not confirm the notion that impaired left ventricular

![Graph](http://hyper.ahajournals.org/)

**Figure 7.** Expression of TGFβ₁ and fibronectin (A) and of ANF mRNA (B) relative to GAPDH. All 3 markers were significantly increased in the hypertrophied left ventricle from the untreated Ren2 rats. Both losartan and tranilast blunted this increase significantly and to a similar extent. SD-TR indicates SD rats treated with tranilast; Ren2TR, Ren2 rats treated with tranilast; Ren2LOS, Ren2 rats treated with losartan.
in agreement with the suggestion that tranilast suspends the cell cycle. We underlined this idea with the in situ analysis, which demonstrated that the mRNA for TGF\(\beta\) was expressed in areas of fibrosis and was hardly found to be expressed in cardiomyocytes but rather was found in areas adjacent to these cells or in the perivascular area. This clearly suggests that noncardiomyocyte cells are largely responsible for the formation of TGF\(\beta\), and may be the most important cell type responsible for the regulation of TGF\(\beta\).

Reduction in TGF\(\beta\) expression failed to prevent left ventricular dysfunction, indicating that other mechanisms may be responsible for the improved survival. TGF\(\beta\) affects many other tissues besides the heart, so one might propose that the described effects could be due to vascular or renal protection. However, tranilast did not prevent thickening of the carotid media (Figure 4), nor did it prevent albuminuria or endothelial dysfunction in this experiment (data not shown). Also in favor of a protective effect on the left ventricle are other recent findings from our group. In a preliminary study, we pretreated rats before experimental myocardial infarction with either tranilast or control food. Tranilast tended to decrease infarction-related mortality rates (from 48% to 16%, \(P=\text{NS}\)) but did not improve left ventricular function (unpublished data).

The improved survival seen in the present study may be due to protection against complications other than loss of contractility, such as arrhythmias. Abundant evidence suggests a relation between cardiac fibrosis and (lethal) cardiac arrhythmias. In hypertensive patients, serum procollagen type III amino-terminal peptide was related to the incidence and severity of arrhythmias.

Losartan and Tranilast Have Strikingly Similar Effects

Losartan and tranilast had strikingly similar effects on most parameters studied. This corroborates the suggestion that a large part of the effects of angiotensin II are mediated via increased expression of TGF\(\beta\). This confirms cell culture data that suggest angiotensin II exerts structural effects through the recruitment of growth factors like TGF\(\beta\).

As can be concluded from the preceding paragraphs, the present study relates the prevention of increased left ventricular TGF\(\beta\) expression to cardiac effects. However, we cannot definitely answer whether the association is causal. Even a nonhypotensive dose of losartan may interfere with many other mechanisms besides angiotensin blockade to attenuate left ventricular hypertrophy. Similarly, tranilast may decrease hypertrophy and mortality rates via undiscovered mechanisms. It is also conceivable that changes in TGF\(\beta\) mRNA translate into complex changes in the amounts of active and latent TGF\(\beta\). Therefore, it remains to be determined how changes in the expression of TGF\(\beta\) translate into changes in TGF\(\beta\) and related peptides. Furthermore, other components of the TGF\(\beta\) system might also be affected.

In the present study, we did not assess the effects of a higher dose of losartan. Recently, we evaluated treatment with losartan started in the Ren2 rat at the same age but in a dose that prevented the development of high blood pressure. There, we demonstrated that this type of treatment prevented all studied changes in the heart. Others have also described this, so it was known at the start of this experiment that normalization of blood pressure with high-dose angiotensin II receptor blockade suffices prevents end-organ changes. We are not the first to show that in this model a nonhypotensive dose of an angiotensin
receptor blocker attenuates the development of left ventricular hypertrophy, although left ventricular function was not assessed in the cited study. In conclusion, it was until now unclear whether chronic in vivo reduction in TGFβ would have beneficial effects in hypertensive heart disease. The present study is the first to suggest that regardless of the mechanism involved, a reduction in TGFβ may be beneficial even in the absence of blood pressure–lowering effects: it attenuated the increase in left ventricular weight, it attenuated the accumulation of collagen, and it improved survival. Surprisingly, a reduction in the expression of TGFβ did not prevent impairment of left ventricular function, so we speculate that a reduction in left ventricular fibrosis may improve survival via an alternative mechanism, such as a reduction in arrhythmias.

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References


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