Tranilast attenuates diastolic dysfunction and structural injury in experimental diabetic cardiomyopathy

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Tranilast attenuates diastolic dysfunction and structural injury in experimental diabetic cardiomyopathy. Am J Physiol Heart Circ Physiol 293: H2860–H2869, 2007. First published August 24, 2007; doi:10.1152/ajpheart.01167.2006.—Diastolic dysfunction is an increasingly recognized complication of diabetes that develops in relatively young patients as a result of diabetic cardiomyopathy (DCM). With recent advances in echocardiographic technology now permitting the reliable assessment of diastolic function in the rat, we examined cardiac function and structure in diabetic rodents and assessed the effects of intervening with tranilast, an antifibrotic compound that has been shown to attenuate the actions of transforming growth factor-β (TGF-β) in cardiac fibroblasts. We also sought to examine the mechanism whereby tranilast inhibits the actions of TGF-β. Six-week-old heterozygous (mRen-2)27 rats were randomized to receive either streptozotocin or citrate buffer and then further randomized to receive either tranilast (400 mg·kg⁻¹·day⁻¹ by twice daily gavage) or vehicle for another 8 wk. Cell signaling was examined in neonatal cardiac fibroblasts. After 8 wk, diabetic rats showed evidence of impaired diastolic function with reduced early-to-late atrial wave ratio and prolonged deceleration time in association with fibrosis, apoptosis, and hypertrophy (all P < 0.05). Treatment with tranilast prevented the development of diastolic dysfunction and the histopathological features of DCM. While tranilast did not affect Smad phosphorylation, it significantly attenuated TGF-β-induced p44/p42 mitogen-activated protein kinase phosphorylation.

THE ABILITY TO NONINVASIVELY assess cardiac function with left ventriculography and echocardiography has brought with it the realization that ∼30–50% of hospitalizations for heart failure occur in patients with preserved left ventricular (LV) systolic function, mostly due to diastolic dysfunction of the LV, a condition to which patients with diabetes are especially prone (47). Indeed, several studies have shown that, well in advance of overt disease, subclinical diastolic dysfunction is present in between 25 and 75% of otherwise healthy normotensive diabetic subjects (4, 35, 51). Given the exclusion of other potential causes of cardiac dysfunction, such as valve disease, hypertension, and coronary artery disease (4), as well as their young age (43), these patients are viewed as having diabetic cardiomyopathy.

While hyperglycemia is a necessary prerequisite for the development of diabetic complications, other components of the diabetic milieu, such as hypertension and the renin-angiotesin system, also contribute. For instance, increased cardiac angiotensin II is a key feature of diabetic cardiomyopathy in humans (11). In contrast to most other rodent models of diabetes, the diabetic (mRen-2)27 rat, like the diabetic human, is hypertensive and shows increase activity of the renin-angiotensin system, developing complications of diabetes that are not seen in other rodent models, such as the spontaneously hypertensive rat, despite similar systemic blood pressures (20). Moreover, in addition to developing diabetic kidney (20) and eye disease (33), the diabetic Ren-2 rat also develops a range of structural abnormalities in the heart that are reminiscent of its human counterpart (29). In the present study, with the availability of rodent echocardiography, we determined the effects of diabetes on cardiac function in the Ren-2 rat and also examined the effects of a novel intervention.

As a clinical entity, diabetic cardiomyopathy is more closely associated with microvascular than macrovascular disease, sharing important structural similarities with diabetic nephropathy that include fibrosis, hypertrophy, and apoptosis (11). These pathological features are likely mediated by locally active growth factors, such as transforming growth factor-β (TGF-β), a key cytokine implicated in the pathogenesis of diabetic complications in both kidney and heart (2, 9, 53), such that blockade of its expression and action represent an important therapeutic target.

A range of agents that inhibit the actions of TGF-β are under investigation for use in fibrotic and also malignant disease (49). Whereas many of these have been specifically developed to target various components of the TGF-β pathway (49), the mechanisms of actions of other agents that interrupt TGF-β-related fibrosis are less well understood. Among this group of antifibrotics is tranilast (n-[3,4-dimethoxycinnamoyl] anthranilic acid), an agent used in Japan for the treatment of exsudative dermal scarring (39, 44) that has also been shown to attenuate the progression of renal dysfunction in patients with diabetic nephropathy (41, 42). In a previous study, our laboratory has shown the ability of tranilast to inhibit TGF-β-induced matrix production in cultured cardiac fibroblasts and reduce cardiac fibrosis in the Ren-2 model of diabetic cardiomyopathy (29). However, whether these structural changes translated into a functional benefit was uncertain, as were the means by which this compound inhibited the actions of TGF-β. Accordingly, the present study had three major aims. First, we...
sought to determine the effects of tranilast on heart function in experimental diabetic cardiomyopathy. Second, although tranilast has been previously shown to reduce fibrosis, its effects on cardiac myocyte apoptosis and hypertrophy have not been examined; therefore, we also sought to study the effects of tranilast on these important pathological feature of diabetic cardiomyopathy. In addition, we also investigated the mechanism by which tranilast might exert its antifibrotic actions, focusing on two interacting cascades that mediate the actions of TGF-β: the Smad and p44/42 mitogen-activated protein kinase (p44/42 MAPK, extracellular signal-regulated kinase) pathways (14).

**MATERIALS AND METHODS**

**Animals and Procedures**

Thirty-two heterozygous (mRen-2) 27 transgenic female (Ren-2) rats were studied. At 6 wk of age, rats were randomized to receive either 55 mg/kg of streptozotocin (Sigma, St. Louis, MO) diluted in 0.1 M citrate buffer, pH 4.5, or citrate buffer (nondiabetic) by tail vein injection following an overnight fast. Each week, rats were weighed, and blood glucose was determined using an Ames glucometer (Bayer Diagnostics, Melbourne, Australia). Diabetic animals received two to four units of isophane insulin (Humulin NPH, Eli Lilly, NSW, Australia) three times per week to promote weight gain and to reduce mortality. At 8 wk of age, both nondiabetic and diabetic rats (n = 8 per group) were further randomized to receive either tranilast (400 mg·kg⁻¹·day⁻¹ by twice daily gavage) or vehicle for another 8 wk. Animals were housed and maintained at a constant room temperature (21 ± 1°C) and 12:12-h light-dark cycle. They were fed standard rat chow and given water ad libitum. Every 2 wk, systolic blood pressure was recorded in preheated conscious rats by tail-cuff plethysmography. Experimental procedures, which adhered to the guidelines of the National Health and Medical Research Council of Australia Code for the Care and Use of Animals for Scientific Purpose and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), were approved for use by the St. Vincent’s Hospital Animal Ethics Committee. At 16 wk of age, animals were anaesthetized with ketamine and xylazine (ketamine 3.75 mg/100 g and xylazine 0.5 mg/100 g without atropine) for echocardiographic studies followed by lethal anesthesia with pentobarbitone sodium (60 mg/kg ip).

**Echocardiography**

Two-dimensional and Doppler echocardiography. Detailed two-dimensional and Doppler echocardiography was performed to assess systolic and diastolic function using a Vivid 7 (GE Vingmed, Horten, Norway) echocardiograph with a 10-MHz phased array probe, as previously published (7). Animals were placed in the left recumbent position and imaged. M-mode echocardiography was performed using a parasternal short-axis view at the level of the papillary muscles. LV posterior and anterior wall thickness were obtained during diastole and systole, as were the LV internal diameter at end-diastole and end-systole. From the parasternal short-axis view, the end-diastolic and end-systolic cross-sectional blood pool areas were measured. Fractional area change (FAC) was then calculated according to the formula: FAC = [(end-diastolic area - end-systolic area)/end-diastolic area] × 100, with end diastole defined as the peak of the R wave, and end systole defined as the end of the T wave.

LV end-diastolic (LVEDV) and end-systolic volumes (LVESV), obtained from the parasternal long-axis view, were calculated according to a single plane area-length method (37). The LV ejection fraction (LVEF) was then calculated by the formula: LVEF = (LVEDV - LVESV)/LVEDV.

Assessment of LV diastolic function was obtained using the apical four-chamber view to assess early and late transmitral peak diastolic flow velocity [early (E) and late atrial (A) waves], using pulsed-wave Doppler with a sample volume of 1 mm placed at the tips of the mitral valve leaflets. Diastolic filling was evaluated by determining the E-to-A ratio (E/A) from the peak velocity of E and A mitral flow and deceleration time (DT). Doppler spectra were recorded for 10 cardiac cycles at a sweep speed of 200 mm/s. All parameters were assessed using an average of three beats, and calculations were made in accordance with the American Society of Echocardiography guidelines. All data were acquired and analyzed by a single, blinded observer using EchoPAC (GE Vingmed) offline processing.

**Circumferential strain rate.** In addition to transmitral flow assessment of diastolic function, circumferential strain and strain rate (SR) were also obtained from parasternal short-axis gray-scale images, using specific rodent software (GE Vingmed, Horten, Norway). Frame rates were optimized from 75 to 110 frames/s. After the endocardium and region of interest were manually defined, a pixel tracking method automatically identified a unique cluster of pixels in the myocardium that could then be tracked through the cardiac cycle, as previously described (16, 36). In brief, maximal integrated strain was calculated in a circumferential plane from the variation in segment length using the tracked end points in each of six LV segments (basal, mid, and apical segments of the septal and lateral walls). The average of the six segments was recorded. SR was calculated as the temporal derivative of integrated strain. Maximal circumferential SR was calculated for each segment in systole, early diastole, and late diastole.

**Histopathology and Immunohistochemistry**

Changes in the aspects of cardiac structure were assessed in a masked protocol, using eight randomly selected tissue sections from each group.

Mean myocyte cross-sectional area was as previously described (48). In brief, myofibers with intact cellular membranes from fields with circular capillary profiles and myofiber shapes were assessed, and the circumferences of 50 cells from the LV were traced and then digitized to calculate mean cross-sectional area.

Collagen subtypes I and III were assessed using goat and mouse anti-collagen-I (Southern Biotechnology Associates, Birmingham, AL) and III antibody (Biogenex, San Ramon, CA). In brief, 4-µm sections were placed into histosol to remove the paraffin wax, dehydrated in graded ethanol, and immersed into tap water before being incubated for 20 min with normal goat serum diluted 1:10 with 0.1 mol/l PBS, pH 7.4. Sections were incubated in respective primary antibodies overnight (18 h) at 4°C. The following day, the sections were thoroughly washed in PBS (3 × 5 min changes), incubate with 3% hydrogen peroxide for 10 min to block endogenous peroxidase, then rinsed with PBS (2 × 5 min), and incubated with either biotinylated swine anti-goat or goat anti-mouse IgG antibody (DAKO, Carpinteria, CA), diluted 1:200 with PBS. Sections were then rinsed with PBS (2 × 5 min) followed by incubation with an avidin-biotin peroxidase complex (Vector, Burlingame, CA), diluted 1:200 with PBS. Following rinsing with PBS (2 × 5 min), localization of the peroxidase conjugates was achieved by using diaminobenzidine tetrahydrochloride as a chromagen for 1–3 min. Sections were rinsed in tap water for 5 min to stop reaction and then counterstained in Mayer’s hematoxylin, differentiated in Scott’s tap water, dehydrated, cleared, and mounted in Depex. Sections incubated with 1:10 normal goat serum, instead of the primary antiserum, served as the negative controls.

The accumulation of immunostaining for collagen I and III was quantified using a method of computer-assisted image analysis that shows a close correlation between immunohistochemical-based quantification and immunoassayable protein content (24, 25). Briefly, 10 random non-overlapping fields from eight rats per group were captured and digitized using a BX50 microscope attached to a Fujix HC5000 digital camera. Digital images were then loaded onto a
Pentium III IBM computer. A threshold value that maximizes the known positive area of brown on immunostained sections (collagen I and III), with minimal background interference, was selected and kept constant during the analysis, as previously described (6). To correct for variation due to shrinkage, the area of positive immunostaining (collagen/mm² tissue) relative to the total area (matrix + myocytes) was determined using computer-assisted image analysis (AIS, Analytical Imaging Station, version 6.0, Ontario, Canada), as previously reported (29).

Apoptosis was examined by quantifying terminal dUTP nick-end labeling, as previously described (19). In brief, sections were deparaffinized and rehydrated in graded ethanol. Permeabilization and removal of endogenous peroxidase was carried out by treating sections with 20 µg/ml proteinase K (Roche Diagnostics, Mannheim, Germany) for 30 min and 3% H₂O₂ for 5 min, respectively. Sections were then washed in dH₂O (2 × 5 min) and treated with TdT buffer (Roche Diagnostics, Mannheim, Germany) for 20 min to allow for equilibration before being treated with TdT enzyme (10 units, Roche Diagnostics) and biotinylated dUTP (1 nmol, Roche Diagnostics) for 60 min at 37°C in a dark, humidified chamber. The reaction was stopped with TB Buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min and washed with PBS (2 × 5 min). Sections were then treated with streptavidin-conjugated horseradish peroxidase (1:100; DAKO) for 30 min at 37°C, washed, incubated with diamino benzidine and counterstained with eosin. Negative control was generated by not treating the section with TdT enzyme, while the positive control was treated with 1 µg/ml DNase I (Promega) in 1 mM MgSO₄/PBS for 20 min before incubation with 3% H₂O₂. The number of positively stained nuclei (brown) per field was then quantified.

**In Vitro Studies**

The effects of tranilast on cell signaling were determined in vitro. Neonatal cardiac fibroblasts were isolated from the hearts of 1-day-old rat pups, purified by differential plating, as previously reported (45), and maintained in DMEM with FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in humidified 5% CO₂ atmosphere at 37°C. Cells at passages 1 or 2 were plated into six-well culture dishes in DMEM/10% FBS at low density and allowed to adhere overnight.

To examine the effects of tranilast on TGF-β signaling, the subconfluent cells were starved overnight in DMEM/0.5% BSA and preincubated with either tranilast (300 nM) or the TGF-β type I receptor kinase inhibitor SB-431542 (10 µM, TOCRIS, Ellisville, MO) for 4 h, followed by stimulation with 10 ng/ml TGF-β₁ (R&D Systems, Minneapolis, MN) for 5 min. To ascertain whether tranilast’s effect on TGF-β-induced extracellular signal-regulated kinase phosphorylation might also apply to other cell types and other stimulatory molecules, we examined the effects of tranilast in kidney mesangial cells and in response to angiotensin II. In brief, a well-characterized cloned rat mesangial cell line (17) (gift of Dr. D. Nikolic-Paterson, Clayton, Australia) was cultured and exposed to TGF-β following 4-h preincubation with either tranilast or 0.9% saline, as described above. To examine the effects of angiotensin II, a potent and rapid inducer of 44/42 MAPK activation, cells were exposed to 100 nM angiotensin II for 5 min following 4-h preincubation with either tranilast or 0.9% saline.

At the end of the incubation, cells were immediately placed on ice, washed once with ice-cold PBS, and lysed in 100 µl of lysis buffer of 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 0.5% Igepal CA-630, 0.1% SDS, 1 mM Na vanadate, 50 mM NaF, 25 mM β-glycerophosphate, 10 mM Na pyrophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. Cell lysates were passed through a 21-G needle five times to aid solubilization. Lysates were placed on ice for 30 min before centrifuging at 13,000 rpm at 4°C to remove cell debris, and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Samples with equal concentrations of protein were subjected to SDS-PAGE and Western blot analysis, as previously described (5), with phosphorylated and total p44/42 MAPK and Smad2 antibodies (1:1,000 in 5% BSA; Cell Signaling, Beverly, CA). Immunoreactive bands were quantified densitometrically, as previously described (5), normalized for total p44/42 MAPK or Smad2, and compared with controls.

### Table 1. Animal characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + Tranilast</th>
<th>Diabetes</th>
<th>Diabetes + Tranilast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>154 ± 9</td>
<td>148 ± 3</td>
<td>166 ± 8</td>
<td>159 ± 11</td>
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<tr>
<td>Glucose, mmol/l</td>
<td>6.3 ± 0.2</td>
<td>6.8 ± 0.3</td>
<td>31.7 ± 0.7</td>
<td>29.8 ± 1.1</td>
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<td>HbA1c, %</td>
<td>3.36 ± 0.03</td>
<td>3.02 ± 0.04</td>
<td>8.46 ± 0.48*</td>
<td>6.19 ± 0.27*</td>
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<tr>
<td>Body weight, g</td>
<td>307 ± 9</td>
<td>299 ± 9</td>
<td>276 ± 6*</td>
<td>274 ± 6*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.18 ± 0.03</td>
<td>0.93 ± 0.03</td>
<td>1.05 ± 0.06</td>
<td>1.09 ± 0.04</td>
</tr>
<tr>
<td>Heart-to-body weight ratio</td>
<td>0.32 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.32 ± 0.01</td>
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</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. control. †Multiplied by 100.

### Table 2. Systolic echocardiographic parameters

<table>
<thead>
<tr>
<th></th>
<th>Ren-2 Control</th>
<th>Ren-2 Control + Tranilast</th>
<th>Ren-2 Diabetic</th>
<th>Ren-2 Diabetic + Tranilast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>206 ± 11</td>
<td>195 ± 15</td>
<td>213 ± 19</td>
<td>222 ± 19</td>
</tr>
<tr>
<td>LVAWd, mm</td>
<td>1.6 ± 0.1</td>
<td>1.7 ± 0.05</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.09</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>1.7 ± 0.05</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.08</td>
<td>1.5 ± 0.06</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>6.7 ± 0.2</td>
<td>6.9 ± 0.2</td>
<td>7.4 ± 0.2*</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>FAC, %</td>
<td>67 ± 3</td>
<td>66 ± 2</td>
<td>72 ± 2</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>LVEDV, µl</td>
<td>269 ± 15</td>
<td>293 ± 23</td>
<td>364 ± 31*</td>
<td>319 ± 26</td>
</tr>
<tr>
<td>LVESV, µl</td>
<td>55 ± 5</td>
<td>64 ± 6</td>
<td>83 ± 10*</td>
<td>74 ± 11</td>
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<tr>
<td>EF, %</td>
<td>79 ± 1</td>
<td>78 ± 2</td>
<td>77 ± 1.4</td>
<td>77 ± 3</td>
</tr>
<tr>
<td>SRs, %</td>
<td>5.8 ± 0.4</td>
<td>6.4 ± 0.4</td>
<td>5.8 ± 0.7</td>
<td>5.1 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. LVAWd and LVPWd, left ventricular anterior and posterior wall thickness in diastole, respectively; LVIDd and LVIDs, left ventricular internal diameter in diastole and systole, respectively; FAC, fractional area change; LVEDV and LVESV, left ventricular end diastolic and systolic volume, respectively; EF, ejection fraction; SRs, circumferential strain rate. *P < 0.05 compared with respective nondiabetic rats.
RESULTS

Animal Characteristics

All rats were hypertensive with elevated systolic blood pressure that was not affected by tranilast (Table 1). Compared...
Fig. 2. Representative sections of immunohistochemistry for types I (A–D) and type III (E–H) collagen in control (A and E), control treated with tranilast (B and F), diabetic (C and G), and diabetic rats treated with tranilast (D and H). In control rats, there was minimal evidence of type I or III collagen immunostaining, whereas diabetic rats were associated with a marked increase in collagen immunostaining. Treatment with tranilast was associated with a reduction in types I and III collagen immunostaining (D). Quantitation of collagen I (I) and III (J) immunostaining in rat heart from control, control treated with tranilast, diabetic, and diabetic rats treated with tranilast is shown. Values are expressed as means ± SE. *P < 0.05 vs. controls. †P < 0.05 vs. diabetic. Magnification ×350.
with control rats, diabetic rats had reduced body weight. Heart weight was unchanged by either diabetes or tranilast (Table 1). HbA1c was elevated in diabetic rats compared with nondiabetic animals that were also unaffected by tranilast.

**Echocardiography**

Echocardiographic data was acquired in all but 4 (2 control, 2 diabetic) of the 32 study animals and demonstrated preserved systolic function across all groups (Table 2). Diastolic function, as assessed by measuring mitral inflow velocity, showed a reduction in the E/A compared with control animals. In contrast, when diabetic rats were treated with tranilast, the E/A was preserved (Table 3, Fig. 1). Assessment of the DT was complimentary to the E/A and demonstrated prolongation in the diabetic group that was normalized by tranilast (Table 3). Evaluation of regional wall motion demonstrated preserved early-diastolic relaxation among all groups. However, compared with nondiabetic animals, late-diastolic velocities were prolonged in the diabetic Ren-2 animals, but not in those diabetic Ren-2 rats that had received tranilast (Table 3, Fig. 1).

**Cardiac Structure**

In comparison with control rats, diabetic rats displayed increased cardiac fibrosis with a greater abundance of interstitial fibrillar collagens I and III (Fig. 2). Diabetic animals also showed evidence of cardiac myocyte hypertrophy (Fig. 3) and an increase in the abundance of terminal dUTP nick-end labeling positive cells (Fig. 4). All of these changes were attenuated by tranilast treatment.

**In Vitro Studies**

Rat cardiac fibroblast cells stimulated with TGF-β1 induced p44/42 MAPK phosphorylation by ~3.5-fold, which was reduced to control levels by treatment with tranilast (Fig. 5). A similar pattern was seen in mesangial cells and in response to angiotensin II. In contrast to the effects of tranilast on TGF-β1-stimulated p44/42 MAPK phosphorylation, the compound had no effect on TGF-β-induced Smad-2 phosphorylation (Fig. 5).

**DISCUSSION**

Diastolic relaxation consists of both an active energy-dependent phase and a passive phase that predominantly reflects the viscoelastic properties of the heart, largely determined by myocardial mass and interstitial collagen. The conventional technique for the assessment of diastolic functions involves the interrogation of transmitral flow during diastole using Doppler echocardiography. As reported in the present study, despite...
normal systolic function, Ren-2 diabetic rats, like humans with diabetic cardiomyopathy (35), had features of impaired relaxation, as evidenced by reduced E/A, prolonged DT, and elevated late-diastolic SR. Without affecting blood pressure or glycemic control, these functional abnormalities were all attenuated by treatment with tranilast.

Diastolic relaxation is a complex process that can be resolved into two separate but interrelated components: an early, energy-dependent active phase during which calcium ions are pumped into the sarcoplasmic reticulum, and a late passive phase that predominantly reflects the viscoelastic properties of the heart and is largely determined by myocardial mass and interstitial collagen (26). Multiple studies have demonstrated abnormal diastolic filling profiles in diabetic patients based on interrogation of transmitral flow during diastole using Doppler echocardiography (35, 51). However, since the assessment provided with this conventional technique for the assessment of diastolic function is to some extent load dependent (12), more recent studies in humans have assessed diastolic function using more sensitive techniques (50), which enable accurate prognostication and differentiation of normal from pseudonormal filling patterns of mitral inflow. These studies report asymptomatic diastolic dysfunction in diabetic subjects (8, 10). In the present study, we, therefore, used a recently rodent-validated echocardiographic technique analogous to the more recent human studies to assess circumferential fiber shortening and relaxation (36). This methodology allows regional wall motion to be assessed and, unlike conventional tissue Doppler-derived strain analysis, is independent of the insonation angle and the effects of cardiac tethering (30). While no apparent change was noted in early-diastolic relaxation among diabetic rats, late relaxation was impaired, demonstrating “a” wave dominance that was consistent with the “impaired pattern” of relaxation demonstrated by spectral Doppler interrogation. Treatment with tranilast was associated with normalization of the spectral Doppler pattern and a reduction in late strain, suggesting improved ventricular compliance, analogous to the pattern of early- and late-mitral annular velocities seen in both normal and impaired relaxation filling patterns (40). These findings are similarly in keeping with the Doppler interrogation and are consistent with the increased collagenous matrix in the hearts of diabetic animals and its attenuation with tranilast.

Fibrosis, hypertrophy, and apoptotic cell death are key events in the development of heart failure that each correlate with the magnitude of cardiac dysfunction (1, 15). As in other forms of heart failure, these histopathological changes are also prominent features of human diabetic cardiomyopathy, in the absence of coronary artery disease (11). Akin to human disease, and as shown in the present study, the Ren-2 rat also undergoes myocyte hypertrophy, fibrosis, apoptotic cell loss, and LV dilatation when diabetes is induced with streptozotocin. Moreover, without affecting blood pressure or glycemic control, tranilast attenuated many of these features of adverse remodeling, consistent with similar findings in other organs (21) and in other disease states (34). Hypertrophy and fibrosis lead to increased heart weight. However, such changes are counterbalanced by increased apoptosis, such that, in the present study, there were no net effects of diabetes on heart

Fig. 4. Terminal dUTP nick-end labeling (TUNEL) in control (A), diabetic (B), and diabetic rats treated with tranilast (C). In control rats (A), only an occasional TUNEL-positive cell was noted, whereas sections from untreated diabetic rats (B) showed numerous positive cells. C: tranilast treatment of diabetic rats was associated with a reduction in the number of TUNEL-positive cells treated. Magnification ×350. D: quantitation of apoptosis by TUNEL in heart sections from control, diabetic, and diabetic rats treated with tranilast. Values are expressed as means ± SE. *P < 0.05 vs. control; †P < 0.05 vs. diabetic.
weight or heart-to-body weight ratio. These findings are at variance to a previous report from our group in which heart-to-body weight ratio was increased in diabetic rats and reduced by tranilast (29). While raw heart weights were not different in any group in either the present report or in our laboratory’s previous published study (29), a statistically significant change was apparent in the latter when indexed to body weight. Between study differences in gross parameters, such as

Fig. 5. Effects of tranilast on intracellular signaling. Tranilast induced inhibition of p44/42 mitogen-activated protein kinase (MAPK) phosphorylation in response to transforming growth factor-β (TGF-β) in cardiac fibroblasts (A and E), kidney mesangial cells (B and F), and in response to angiotensin II (ANG II; C and G). D: in contrast, TGF-β-induced Smad2 phosphorylation in cardiac fibroblasts was unaffected by tranilast. In the quantitative analysis (E–G), values are expressed as that ratio of phosphorylated to total p44/42 MAPK as means ± SE. AU, arbitrary units. *P < 0.01 vs. control, †P < 0.01 vs. TGF-β1 or ANG II.
heart-to-body weight ratio, may be due to a wide range of factors that are commonly reported in outbred rat strains, such as the Sprague-Dawley and Ren-2 rats. These include not only genetic variations but also the inevitable variability in glycemic control and blood pressure. More robust assessments of organ structure, however, such as cell hypertrophy, matrix deposition, and apoptosis, as used in the present report are, however, quite consistent with previous studies in this rodent model. In the present study, LV dilatation in diabetic animals was not improved with tranilast, despite reductions in fibrosis, apoptosis, and hypertrophy. The mechanisms underlying this incomplete response are uncertain. However, the absence of an effect on blood pressure and consequently afterload, contrasting the actions of an ACE inhibitor, may account, at least in part, for the persistence of LV dilatation with tranilast.

In addition to the renin-angiotensin-aldosterone and sympathetic-adrenal systems, the growth factor TGF-β has also been consistently implicated in the pathogenesis of fibrosis, apoptosis, and hypertrophy in the heart (34, 38), as in other organs (3). Indeed, the pathogenetic effects of TGF-β are exacerbated by a high-glucose environment (29). Accordingly, antagonizing the effects of TGF-β represents an important therapeutic target in heart disease (15). In addition to the range of agents that have been strategically designed to target specific aspects of the TGF-β cascade (49), there are also a group of compounds with demonstrated TGF-β inhibitory actions for which a mechanism of action is less clear. These agents include several orally active, chemically dissimilar, anti-fibrotic compounds, such as pirfenidone, pentoxifylline, and tranilast, that are currently being evaluated in both the preclinical and clinical settings (13, 27, 31, 32, 41). For tranilast, previous studies have shown that it reduces TGF-β-induced matrix production in a range of cell types, including cardiac fibroblasts, and that it also attenuates pathological fibrosis in the kidney and heart in a variety of settings, including a high-glucose environment (29, 32). However, while this study focused on the role of extracellular matrix in the pathogenesis of diastolic dysfunction in diabetes, a range of other perturbations in cardiac function may also contribute to impaired relaxation, including alterations in the myocyte calcium handling proteins, SERCA-2a and phospholamban, as well as changes in phenotype of contractile proteins and their regulatory elements (18).

Having demonstrated its ability to attenuate TGF-β-induced matrix production, we further sought to explore the mechanisms underlying this effect. In the classical pathway of TGF-β signaling, ligand-receptor binding leads to phosphorylation of the receptor-activated Smad2, leading to a cascade of changes in other Smads that ultimately translocate to the nucleus to initiate the transcription of target genes (52). In the present study, Smad2 phosphorylation was unaffected by tranilast, contrasting the effects of a TGF-β type I receptor kinase inhibitor. However, while tranilast did not inhibit TGF-β-induced Smad2 phosphorylation in vitro, an attenuation in the expression of this downstream mediator was noted in vivo, in a previous publication from our group (29). These seemingly disparate results are likely due to the time-dependent ability of TGF-β to positively regulate its own expression (22, 46). This well described auto-induction involves gene transcription and requires at least 3 h of exposure to TGF-β before becoming detectable (46). This was evident in our laboratory’s previous report (29), whereby, in long-term studies, tranilast reduced the abundance of TGF-β and thus also the magnitude of phospho-Smad2. Therefore, to investigate the effects of tranilast on TGF-β cell signaling, in the present study, cells were exposed to TGF-β for only 5 min, well before the confounding effect of auto-induction might become apparent.

The effects of TGF-β, like many other peptides, are not mediated through a single intracellular signaling pathway. For instance, in addition to the Smad, TGF-β also induces matrix expression through the p44/42 MAPK pathway (23). Accordingly, we examined the latter and noted a diminution in TGF-β-induced p44/42 MAPK phosphorylation in the presence of tranilast, suggesting that modulation of this pathway may explain, at least in part, tranilast’s ability to inhibit the actions of TGF-β. These effects were also seen in kidney mesangial cells and in response to angiotensin II, another inducer of p44/42 MAPK phosphorylation. In contrast to its actions on p44/42 MAPK phosphorylation, tranilast did not affect TGF-β-induced phosphorylation of Smad2.

In summary, tranilast reduced both the functional and structural abnormalities in the diabetic Ren-2 rat, by antagonizing the effects of TGF-β. While inhibition of p44/42 MAPK phosphorylation was noted, the precise mechanisms of tranilast’s actions remain to be elucidated. However, in the context of recent studies in both early and later stage human diabetic nephropathy (41, 42), the findings of the present report suggest that tranilast may also be of potential benefit in diabetic cardiomyopathy.

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REFERENCES
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. Hypertension 36: 747–754, 2000.


40. Sohn DW, Chai HL, Lee DJ, Kim HC, Kim HS, Oh BH, Lee MM, Park YB, Choi YS, Seo JD, Lee YW. Assessment of mitral annulus velocity by Doppler tissue imaging in the evaluation of left ventricular diastolic dysfunction. J Am Coll Cardiol 30: 474–480, 1997.


