Distinct cardiac and renal effects of ET$_A$ receptor antagonist and ACE inhibitor in experimental type 2 diabetes

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Zoja C, Cattaneo S, Fiordaliso F, Lionetti V, Zambelli V, Salio M, Corna D, Pagani C, Rottoli D, Bisighini C, Remuzzi G, Benigni A. Distinct cardiac and renal effects of ET$_A$ receptor antagonist and ACE inhibitor in experimental type 2 diabetes. Am J Physiol Renal Physiol 301: F1114–F1123, 2011. First published August 3, 2011; doi:10.1152/ajprenal.00122.2011.—Diabetic nephropathy is associated with cardiovascular morbidity. Angiotensin-converting enzyme (ACE) inhibitors provide imperfect renoprotection in advanced type 2 diabetes, and cardiovascular risk remains elevated. Endothelin (ET)-1 has a role in renal and cardiac dysfunction in diabetes. Here, we assessed whether combination therapy with an ACE inhibitor and ET$_A$ receptor antagonist provided reno- and cardioprotection in rats with overt type 2 diabetes. Four groups of Zucker diabetic fatty (ZDF) rats were treated orally from 4 (when proteinuric) to 8 mo with vehicle, ramipril (1 mg/kg), sitaxsentan (60 mg/kg), and ramipril plus sitaxsentan. Lean rats served as controls. Combined therapy ameliorated proteinuria and interstitial inflammation. Cardiomyocyte loss, volume enlargement, and capillary rarefaction were prominent abnormalities of ZDF myocardium. Myocyte volume was reduced by ramipril and sitaxsentan, which also ameliorated heart capillary density. Drug combination restored myocardial structure and reestablished an adequate capillary network in the presence of increased cardiac expression of VEGF/VEGFR-1, and significant reduction of oxidative stress. In conclusion, in type 2 diabetes concomitant blockade of ANG II synthesis and ET-1 biological activity through an ETA receptor antagonist led to substantial albeit not complete renoprotection, almost due to the ACE inhibitor. The drug combination also showed cardioprotective properties, which however, were mainly dependent on the contribution of the ET$_A$ receptor antagonist through the action of VEGF.

Zucker diabetic fatty rats; endothelin-1; VEGF/VEGFR-1

Type 2 diabetes, which accounts for 90–95% of all diagnosed cases of diabetes, is dramatically increasing worldwide as a result of increased obesity, older age, urbanization, sedentary lifestyle, and stress (57). The prevalence of diabetes-associated complications, including nephropathy, cardiovascular (CV) disease, retinopathy, and neuropathy, will soon constitute an enormous burden for healthcare systems. Both diabetes and chronic kidney disease are risk factors for CV disease. For type 2 diabetic patients, the risk of CV disease is two to three times higher than for the general population (22). Large community-based studies have documented an increased risk of CV disease and all-cause mortality with decreasing estimated glomerular filtration rate (GFR) (26). In type 2 diabetes, microalbuminuria is a marker of renal dysfunction and a strong and independent predictor of CV disease that carries prognostic information even in ranges that are currently considered normal (45). Microalbuminuria and CV disease are both expressions of a common underlying disorder characterized by endothelial dysfunction and/or chronic low-grade inflammation (45, 52). Thus intensive treatment to target microalbuminuria is warranted in diabetic patients both for reno- and cardioprotection. Previous studies have shown that drugs which block ANG II synthesis/biological activity in addition to prevent micro- (44) or macroalbuminuria limited CV events in subjects with diabetes (8). However, ANG II blockers failed to limit the advanced phase of diabetes (39), suggesting the need of a multidrug renoprotective strategy to contemporarily inhibit diverse pathogenetic pathways.

Besides ANG II, endothelin-1 (ET-1) has been found to exert a central role in endothelial dysfunction in type 2 diabetes (42). Altered sensitivity to exogenously infused ET-1 has been reported (11), together with the presence of elevated plasma levels of ET-1 in type 2 diabetic patients (15). Renal function impairment and progression to CV events were associated with elevated plasma levels of the endothelin precursor COOH-terminal proET-1 in patients with type 2 diabetes (34). Critical involvement of ET-1 in renal and cardiac dysfunction in diabetes has been suggested by findings of enhanced urinary excretion of ET-1, which reflects the renal synthesis of the peptide, in both experimental and clinical settings (35, 36), and increased cardiac ET-1 mRNA and protein levels (29, 32).

We previously demonstrated that concomitant administration of an ACE inhibitor and ET$_A$ receptor antagonist offered better renoprotection than single agents in rats with advanced type 1 diabetes (25), but no data are available for type 2 diabetes. Type 2 diabetes animal models can differ in genetic pathways.

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rats, male ZDF rats progress to frank diabetes due to failure to compensate adequately for insulin resistance. ZDF rats are less obese but more insulin resistant than Zucker rats; they are characterized by hyperlipidemia, altered metabolic profile, progressive renal injury, and cardiac abnormalities, which make them a model of severe type 2 diabetes.

The present study was designed to evaluate the effect of combined therapy with an ACE inhibitor and ETA receptor on diabetes-associated renal abnormalities and cardiac damage in ZDF with advanced disease.

MATERIALS AND METHODS

Experimental animals. Animal care and treatment were conducted according to institutional guidelines in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale suppl 40, 18 febbraio 1992, Circolare n.8, Gazzetta Ufficiale 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL385-1, December 1987; Guide for the Care and Use of Laboratory Animals; US National Research Council, 1996). Animal studies were submitted to and approved by the Institutional Animal Care and Use Committee of “Mario Negri” Institute, Milan, Italy. Two month-old male ZDF rats (ZDF/Gmi-fa/fa) and aged-matched nondiabetic lean rats (ZDF/Gmi-fa/-); Charles River Laboratories Italia S.r.l., Calco, Italy) were kept on a 12:12-h light-dark cycle with free access to water. ZDF rats were maintained on Purina 5008 rat chow (26.8 kcal% protein, 56.4 kcal% carbohydrate, 16.7 kcal% fat) to accelerate the onset of diabetes. At 4 mo of age, ZDF rats were randomized to receive the following daily until month 8 (n = 8/group): vehicle (by gavage); ramipril (1 mg/kg in the drinking water); ETα receptor antagonist sitaxsentan was chosen on the basis of previous experiments (data on file, Pfizer). Seven lean rats were used as controls.

Biochemical and hemodynamic parameters. Blood glucose levels were assessed with a reflectance meter (Ascensia Elite, Bayer, Milan, Italy). Serum cholesterol, triglycerides, and blood urea nitrogen (BUN) were measured by a Reflotron test (Roche Diagnostic, India). Serum glucose, cholesterol, triglycerides, and blood urea nitrogen (BUN) were measured by a Reflotron test (Roche Diagnostics, Indianapolis, IN). Albuminuria was measured by ELISA (Nephrat II ELISA, Exocell, Philadelphia, PA). Heart rate and systolic blood pressure (SBP) were measured with a computerized tail-cuff system in conscious rats (BP-2000 Blood Pressure Analysis System, Visitech System, Apex). The rate-pressure product (RPP; RPP = HR·SBP), an index of cardiac metabolism (12), was calculated.

Renal histology and immunohistochemical analysis. Kidneys were fixed overnight in Dubosq-Brazil, dehydrated in alcohol, and embedded in paraffin. Three-micrometer sections (Ultrotome V, LKB, Bromma, Sweden) were stained with hematoxylin and eosin and periodic acid-Schiff reagent. At least 100 glomeruli, including superficial and juxamedullary cortical area, were examined for each animal. The extent of glomerular damage was expressed as the percentage of sclerotic glomeruli. Renal biopsies were simultaneously processed and analyzed by the same pathologist, who was unaware of the nature of the experimental groups. ET-1 and type III collagen were detected by the ABC immunoperoxidase method on paraffin-embedded tissue. Monoclonal mouse anti-rat ET-1 antibody (1:250, Abcam, Cambridge, UK) and polyclonal rabbit anti-rat type III collagen antibody (1:100, Chemicon, Temecula, CA) were used. Visualization was performed by dianinobenzidine. The signal intensity was graded on a scale of 0 to 3: 0, no staining; 1, weak staining; 2, staining of moderate intensity; and 3, strong staining. The score of collagen deposition was determined in at least randomly selected 20 fields at ×400 magnification.

Mouse monoclonal antibody (Chemicon) was used for the detection of monocyte/macrophage ED-1 surface antigen by the alkaline phosphatase-fast red technique. Positive cells were counted on an average of 35 randomly selected interstitial high-power microscopic fields (×400) for each animal.

Cardiac histology and ultrastructure. Sections of left ventricle (LV) were cut perpendicularly to its longitudinal axis and fixed in phosphate-buffered 4% formaldehyde for paraffin-embedded sections or in 3% glutaraldehyde for electron microscopy analysis. LV sections were incubated with rhodamine-Griffonia simplicifolia lectin I (1:50) for capillary density determination. Cross-sectional capillary profiles were then counted at ×600 magnification at different levels along the longitudinal axis of the heart using a fluorescence microscope (Olympus IX51).

Interstitial collagen was assessed in 0.1% sirius red-stained LV sections and quantified at a final ×200 magnification with a microscope (Axioskope, Zeiss) connected to a video camera (XC-77CE CCD Video Camera Module, Sony). The resulting image was processed on the Kontron KS500 image-analysis system (Kontron-Zeiss). The content of interstitial collagen (expressed as the fractional area of the entire cross section) was averaged on nine fields selected across the wall thickness in the septum and free wall. The nature of the sirius red-stained collagen deposit was confirmed by examining the sections under a microscope fitted with a linear cross-polarizing filter that renders collagen fibers birefringent. Perivascular fibrosis was examined in 15–20 coronary arterioles (lumen diameter <100 μm) in each LV after sirius red staining.

For ultrastructure analysis, LV samples were postfixed for 2 h in OsO4. After dehydration in graded series of ethanol, tissue samples were cleared in propylene oxide, embedded in Epoxy medium (Epon 812 Fluka), and polymerized at 60°C for 72 h. Ultrathin (30- to 70-nm thick) sections of areas of interest were obtained with a Leica EM-UC6 ultramicrotome, counterstained with uranyl acetate and lead citrate, and examined with an Energy Filter Transmission Electron Microscope (EFTEM, Zeiss Libra 120) equipped with a YAG scintillator slow-scan CCD camera. Subsarcolemmal and interfibrillar mitochondria were observed at a magnification of ×20,000 in two nonadjacent sections of each sample. Damage of mitochondria, characterized by massive swelling, separation of cristae, and/or inner membrane ruptures, were expressed as the percentage of the total mitochondria analyzed (n = 948–1,140/sample).

Morphometric determination of myocyte number and volume. Number and volume of myocytes were determined by a quantitative morphometric method on LV sections stained with hematoxylin and eosin, as previously described (6).

Immunohistochemistry for heart nitrotyrosine staining. Paraffin-embedded heart sections (3 μm) were processed as described (6). Ten to 20 fields (×200 magnification) for each section were analyzed, and scoring (0, absent; 1, faint; 2, moderate; 3, intense) of nitrotyrosine staining was calculated as a weighed mean.

Quantitative real-time PCR. Total RNA was isolated from kidney and heart tissue using TRIzol reagent (Invitrogen). Purified RNA (2 μg) was reverse transcribed. Amplification was performed on the 7300 RT-PCR System using TaqMan Universal PCR Master Mix (Applied Biosystems) and inventoried TaqMan assays: Rn00561129_m1 for rat ET-1, Rn00580555_m1 for rat monocyte chemoattractant protein (MCP)-1, Rn01511602_m1 for rat VEGFA, Rn 00570815_m1 for rat VEGFR-1 (target genes), with universal (18S) and endogenous control (VIC/MGB probe) as the reference gene. The ΔΔCT technique was used to calculate relative changes in expression of target genes with respect to a calibrator sample (control lean rat).

Statistical analysis. Results are means ± SE. Data were analyzed using ANOVA coupled with Bonferroni post hoc analysis or...
RESULTS

**ET-1 gene and protein expression.** The expression of ET-1 mRNA was evaluated by RT-PCR in whole kidney homogenate of diabetic and lean rats at month 8. As shown in Fig. 1A, the mRNA levels of ET-1 were enhanced in ZDF rats compared with lean rats (P < 0.01). Immunohistochemical analysis revealed that ET-1 protein was increased in the kidney of ZDF rats with respect to lean rats (Fig. 1B). The ET-1 protein staining mainly localized in tubular epithelial cells. A few glomerular cells, possibly podocytes, were also positive for ET-1.

**Body weight and survival.** ZDF rats gained weight during the study and were heavier than lean rats at 4 mo of age (392 ± 3 vs. lean 365 ± 6 g, P < 0.01). With time, their body weights tended to stabilize, while those of lean rats continued to increase. At 8 mo, ZDF rats had lower body weights than lean rats. This observation was in agreement with previous studies (18, 37, 49). Body weights of diabetic rats receiving ramipril or sitaxsentan were comparable with those given vehicle while animals on the combined therapy grew less than vehicle-treated rats. This observation was in agreement with previous studies (18, 37, 49). Body weights of diabetic rats receiving ramipril or sitaxsentan were comparable with those given vehicle while animals on the combined therapy grew less than vehicle-treated rats. This observation was in agreement with previous studies (18, 37, 49).

**Laboratory tests.** Blood glucose levels of all ZDF rat groups were significantly higher than those of lean rats at 8 mo of age. Ramipril or sitaxsentan alone or their combination did not affect blood glucose (Table 1).

Serum cholesterol in ZDF rats given vehicle was increased (P < 0.01) compared with lean rats; ramipril ameliorated the lipid profile of diabetic rats, while sitaxsentan had no effect. The reduction of hypercholesterolemia obtained with combined therapy was due to the effect of ramipril (Table 1). Diabetic rats on vehicle showed higher serum triglyceride levels (P < 0.01) than lean rats. Serum triglycerides were significantly lessened by ramipril but not by sitaxsentan. Ramipril plus sitaxsentan were more effective than single drugs in reducing hypertriglyceridemia of diabetic rats (Table 1).

**Renal parameters.** The time course of albuminuria is shown in Fig. 2. In ZDF rats given vehicle, albuminuria levels were higher than in lean rats at any time point during the study (P < 0.01). Albuminuria was decreased by ramipril starting from 5 mo of age (P < 0.01 vs. vehicle) and reached 57% reduction at the end of the study. Sitaxsentan significantly reduced albuminuria only at 5 mo. The combined therapy showed a significant albuminuria-lowering effect (P < 0.01 vs. vehicle), reaching 72% reduction at 8 mo.

Serum BUN, was mildly, although significantly, increased in ZDF rats on vehicle (38 ± 5 vs. lean 22 ± 1.5 mg/dl; P < 0.01). BUN levels were reduced only by combined therapy (26 ± 4 mg/dl, P < 0.05 vs. vehicle).

**Renal structural changes.** ZDF rats on vehicle developed glomerulosclerosis affecting 20% of glomeruli (Fig. 3A). Ramipril decreased the incidence of glomerulosclerosis to 10%, while sitaxsentan did not modify glomerulosclerosis. After combined therapy, glomerulosclerosis in diabetic rats was decreased to 8% (P < 0.05 vs. vehicle).

Diabetic rats given vehicle exhibited interstitial fibrosis as shown by increased staining of type III collagen in respect to

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**Table 1. Systemic parameters measured in lean and diabetic rats at 8 mo**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Blood Glucose, mg/dl</th>
<th>Serum Cholesterol, mg/dl</th>
<th>Serum Triglycerides, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lean</td>
<td>454 ± 8</td>
<td>126 ± 11</td>
<td>129 ± 3</td>
<td>169 ± 13</td>
</tr>
<tr>
<td>ZDF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>412 ± 8</td>
<td>521 ± 17</td>
<td>446 ± 49</td>
<td>1,890 ± 293</td>
</tr>
<tr>
<td>Ramipril</td>
<td>396 ± 14</td>
<td>530 ± 28</td>
<td>288 ± 19</td>
<td>883 ± 199</td>
</tr>
<tr>
<td>Sitaxsentan</td>
<td>390 ± 11</td>
<td>538 ± 18</td>
<td>427 ± 37</td>
<td>1,438 ± 235</td>
</tr>
<tr>
<td>Ramipril + sitaxsentan</td>
<td>377 ± 7</td>
<td>492 ± 28</td>
<td>242 ± 6</td>
<td>587 ± 91</td>
</tr>
</tbody>
</table>

Values are means ± SE. ZDF, Zucker diabetic fatty rat. *P < 0.01 vs. control lean. **P < 0.05, ***P < 0.01 vs. vehicle. 1*P < 0.05, 2*P < 0.01 vs. sitaxsentan.
Lean rats (Fig. 3B). Ramipril and the combined therapy significantly ($P < 0.05$) reduced type III collagen deposition. Sitaxsentan lowered protein matrix accumulation, although statistical significance was not achieved.

**Effect of treatments on renal inflammation.** An increased number of ED-1$^+$ monocytes/macrophages were observed in the renal interstitium of ZDF rats given vehicle compared with lean rats (Fig. 4A). Ramipril reduced by 82% the interstitial accumulation of monocytes/macrophages ($P < 0.01$ vs. vehicle). Fewer infiltrates were also found after sitaxsentan (62% reduction vs. vehicle, $P < 0.05$). Combined therapy reduced the accumulation of monocytes/macrophages to normal values (Fig. 4A).

Based on the above anti-inflammatory effect of treatments, we next evaluated renal MCP-1 mRNA (Fig. 4B). A twofold increase in chemokine expression was found in vehicle-treated rats compared with lean rats. Both ramipril and sitaxsentan reduced MCP-1 levels with respect to vehicle. The drug combination normalized MCP-1 expression.

**Cardiac parameters.** Heart rate was significantly reduced in ZDF rats compared with lean rats, independently of treatment (Table 2). ZDF rats given vehicle showed mild hypertension (Fig. 5). The ACE inhibitor reduced SBP at levels lower than those of the vehicle group ($P < 0.01$). Sitaxsentan induced a transient, significant ($P < 0.05$) reduction of SBP at 5 and 6 mo. At 8 mo, SBP levels remained numerically lower than those of vehicle, but statistical significance was not achieved. Combined therapy ameliorated SBP at the same extent as ramipril for the entire study period (Fig. 5). RPP was significantly reduced in ZDF rats treated with ramipril and ramipril plus sitaxsentan (Table 2), suggesting that myocardial oxygen demand was not increased during long-term treatment.

**Cardiac structural changes.** Compared with lean rats, LV weight was lower in ZDF rats. Single treatments and the drug combination did not affect LV weight (Table 2). The total number of cardiomyocytes in the LV of ZDF rats was reduced by 21% ($P < 0.01$) compared with lean rats (Fig. 6A). Neither single treatments nor the combination of ramipril and sitaxsentan was capable of limiting myocyte loss in ZDF rats. A marked enlargement of cardiomyocyte volume was observed in ZDF vs. lean rats ($P < 0.01$) (Fig. 6B). Ramipril significantly reduced cardiomyocyte volume of ZDF rats, while treatment with sitaxsentan showed a numerical reduction. Myocyte hypertrophy was completely reversed by combination therapy.

Cardiac microvascular density was reduced by 24% ($P < 0.01$) in ZDF rats given vehicle vs. lean rats (Fig. 7). Ramipril did not exert any proangiogenic effect on ZDF rats, while sitaxsentan significantly increased the production of new capillaries in ZDF myocardium with respect to animals receiving vehicle or ramipril. The combination of ramipril and sitaxsentan restored the number of capillaries to levels comparable to those of lean rats. Intersitial collagen calculated by sirius red staining or volume occupied by myocytes in LV myocardium was not different in lean and ZDF rat groups (data not shown).

Alterations of myocardium of ZDF rats on vehicle were evident only at the ultrastructural level, with disarrangements of myofibrils and sarcomere fragmentation (white arrows, Fig. 8). Mitochondria underwent degenerative processes of different severity, leading in some cases to complete disruption of internal components as observed in ZDF rats treated with vehicle and ramipril (black arrows). Mitochondrial damage was limited to some areas of the inner membrane in ZDF rats treated with sitaxsentan (black arrows). Quantitative assessment of damaged mitochondria revealed that the percentage of damaged mitochondria was significantly reduced by ramipril ($P < 0.05$) and sitaxsentan ($P < 0.01$) with respect to vehicle-treated animals but normalized by the drug combination (Fig. 8). Organization

![Graph](image-url)
of the contractile apparatus was preserved in the myocardium of ZDF rats treated with combination therapy, with the presence of few slightly damaged mitochondria (black arrows, Fig. 8).

**VEGF and VEGFR-1 expression.** To assess the mechanisms underlying the favorable effect of combined therapy on myocardial microvascular architecture, we studied the expression of VEGF and its receptor VEGFR-1 by RT-PCR (Fig. 9). VEGF expression was comparable in the heart of ZDF and lean rats. Ramipril did not affect transcript levels, while sitaxsentan significantly (P < 0.05) increased VEGF expression compared with vehicle-treated rats. The drug combination further increased the expression of the angiogenic factor (P < 0.01 vs. vehicle). In addition, all pharmacological treatments were capable of enhancing the LV expression of VEGFR-1. Sitaxsentan and the combined therapy induced an increase in VEGFR-1 expression significantly higher than that observed with ramipril (Fig. 9).

**Oxidative damage of cardiomyocytes.** An excess formation of peroxynitrite, the reaction product of nitric oxide and superoxide anion, was taken to reflect increased lipid peroxidation and oxidation of structural proteins (56). Expression of nitrotyrosine, a marker of peroxynitrite production, was increased in cardiomyocytes from vehicle-treated rats compared with lean rats (Fig. 10). Ramipril did not affect nitrotyrosine

### Table 2. Hemodynamic recordings in lean and diabetic rats at 8 mo

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart Rate, beats/min</th>
<th>Rate Pressure Product (mmHg × beats/min)10³</th>
<th>Left Ventricle Weight, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lean</td>
<td>388 ± 16</td>
<td>53 ± 2</td>
<td>1,051 ± 20</td>
</tr>
<tr>
<td>ZDF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>315 ± 11†</td>
<td>45 ± 2*</td>
<td>964 ± 32*</td>
</tr>
<tr>
<td>Ramipril</td>
<td>315 ± 10†</td>
<td>37 ± 2†</td>
<td>956 ± 27*</td>
</tr>
<tr>
<td>Sitaxsentan</td>
<td>308 ± 4†</td>
<td>41 ± 1*</td>
<td>915 ± 33†</td>
</tr>
<tr>
<td>Ramipril + sitaxsentan</td>
<td>308 ± 16*</td>
<td>36 ± 3†</td>
<td>898 ± 39*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, †P < 0.01 vs. control. ‡P < 0.01 vs. vehicle.
expression, while sitaxsentan and the combined therapy reduced nitrotyrosine staining with respect to vehicle- and ramipril-treated rats ($P < 0.05$).

**DISCUSSION**

The inbred obese ZDF rats, that carry the Lepr$^{fa}$ mutation, develop non-insulin-dependent diabetes and progressive renal disease in association with cardiac abnormalities (4, 23), which renders ZDF rats a suitable model for studying renal and cardiovascular alterations in type 2 diabetes. The first finding of the present study was that ZDF rats had higher renal expression of ET-1 mRNA and protein compared with lean rats. This, together with the observation of increased transcript levels of ET-1 mRNA in the heart of ZDF rats (29), represented the rationale for targeting the ET system to induce simultaneous reno- and cardioprotection in type 2 diabetes.

Kidney cells and cardiomyocytes produce ET-1 (42, 50), which acts upon binding to ET A and ETB receptors. Here, we elected to use a selective ETA receptor antagonist given the protective role of ETB receptor in the kidney (55), and the evidence that ETB-deficient diabetic rats develop a more severe disease than wild-type littermates (40). The choice was also based on human data of more favorable effects of ETA compared with ET A/ ETB receptor antagonists on renal hemodynamics, at comparable blood pressure control (27).

ZDF rats developed progressive albuminuria, which was partially reduced by ramipril. Sitaxsentan only transiently reduced albuminuria possibly as a consequence of the concomitant significant reduction of blood pressure, in line with studies in experimental type 1 diabetes (25). In different experimental models of renal diseases, the blood pressure-lowering effect of ET receptor antagonists (at different degrees depending on the animal model and employed drugs) was associated with changes in proteinuria/albuminuria (5, 7, 17, 25, 48). Scanty evidence are instead available indicating that ETA blockade displayed a blood pressure-independent effect on progressive renal injury (2, 54). Several variables including the animal model, the time at which treatment started, and drug selectivity could explain such discrepant results. Proteinuria is the consequence of either impairment of glomerular membrane size selectivity or increased glomerular capillary pressure. ET-1 has been found to impact glomerular permeability in normal (46) and type 1 diabetic isolated glomeruli (47) via the ETA receptor. However, direct in vivo evidence obtained by fractional clearance of graded-size Ficoll molecules challenged these results. Thus altered glomerular size selectivity to large macromolecules in diabetic rats, while remarkably improved by an ACE inhibitor, was not changed by an ETA receptor antagonist, ruling out that the latter drug class could affect the glomerular sieving properties (25). Whether ET receptor antagonists might reduce glomerular capillary hypertension has never been assessed so far, and micropuncture studies are warranted to establish this potential drug capability in experimental animals. In support of a renal hemodynamic mechanism underlying the reduction of proteinuria are data in patients with chronic renal disease treated with sitaxsentan (16). In these patients, the fall in filtration fraction after an ETA receptor antagonist was interpreted as the drug’s effect of preventing the efferent, more than the afferent, arteriolar constriction induced by

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**Fig. 6.** Effect of treatment with ramipril, sitaxsentan, and ramipril plus sitaxsentan on myocyte number (A) and volume (B) in ZDF rats at month 8. Values are means ± SE. $^*P < 0.05$, $^{**}P < 0.01$ vs. control lean. $^*P < 0.01$ vs. vehicle. $^{#}P < 0.05$ vs. ramipril. $^{#}P < 0.05$ vs. sitaxsentan.

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**Fig. 7.** Cardiac capillary density in diabetic animals after vehicle, ramipril, sitaxsentan, or combined therapy at month 8. Capillary density was assessed by counting at high-power fields ($\times 600$) the red fluorescent cross-sectional capillary profiles in 20 fields of left ventricular sections stained with rhodaminated Griffonia simplicifolia lectin I. Values are expressed as capillaries/mm$^2$ (means ± SE). $^*P < 0.05$, $^{**}P < 0.01$ vs. control lean. $^*P < 0.05$, $^{**}P < 0.01$ vs. vehicle. $^{#}P < 0.05$, $^{##}P < 0.01$ vs. ramipril.
ET-1 via the ET\textsubscript{A} receptor. The postulated reduction of efferent arteriolar tone with sitaxsentan should reduce glomerular perfusion pressure, resulting in less proteinuria associated with a fall in GFR that was observed after an ETA receptor antagonist. Here, the effect of the combination therapy of an ACE inhibitor and ETA receptor antagonist on albuminuria mostly depended on the action of ramipril, but was not complete. This translated into a partial amelioration of glomerulosclerosis and interstitial fibrosis, which indicates the need for a further compound to be added to the two to provide complete protection.

Inflammation plays a key role in the pathogenesis of type 1 and type 2 diabetic nephropathy. Attenuation of renal macrophage infiltration was reported in type 1 diabetes rats given an ET\textsubscript{A} receptor blocker (48). This also occurs in type 2 diabetes as sitaxsentan inhibited the chemoattractant signal evoked by MCP-1, leading to less accumulation of mononuclear cells in renal interstitium. Simultaneous interruption of ANG II and ET-1 pathways was even more effective than single drugs as it normalized MCP-1 levels and abrogated renal inflammation. Our data showing an anti-inflammatory but not antiproteinuric effect of sitaxsentan indicate that the ETA receptor antagonist acts to limit the deleterious consequences of proteinuria rather than proteinuria itself. Abnormally filtered proteins activate proximal tubular cells to release into the renal interstitium ET-1

![Ultrastructure analysis of myocardium](image)

**Fig. 8. Ultrastructure analysis of myocardium.** Transmission electron micrographs of myocardium of lean and diabetic rats treated with vehicle, ramipril, sitaxsentan, or ramipril+sitaxsentan. Note sarcomere fragmentation and damaged mitochondria in vehicle- and ramipril-treated animals, and preservation of contractile apparatus and restoration of normal structure in almost every mitochondrion after ramipril+sitaxsentan. Graph represents percentage of damaged mitochondria in myocardium of lean and ZDF rat groups. Quantification of damaged mitochondria was performed by transmission electron microscopy analysis at ×8,000. Values are means ± SE. **P < 0.01 vs. control lean. *P < 0.05, **P < 0.01 vs. vehicle. #P < 0.05 vs. ramipril. ΔP < 0.05 vs. sitaxsentan.

![mRNA expression of VEGF and its receptor VEGFR-1 assessed at month 8 in the hearts of diabetic rats](image)

**Fig. 9.** mRNA expression of VEGF and its receptor VEGFR-1 assessed at month 8 in the hearts of diabetic rats after vehicle, ramipril, sitaxsentan, or combined therapy. Values are means ± SE. *P < 0.05 vs. vehicle, *P < 0.01 vs. control lean and ZDF on vehicle. #P < 0.05 vs. ramipril.
and chemokines. These promote local recruitment of inflammatory cells, which further generate chemoattractants and growth factors that amplify the inflammatory reaction (1).

Dyslipidemia in ZDF rats was significantly limited by ramipril and the combined therapy, but not sitaxsentan. The lipid-lowering effect of ramipril is likely the consequence of the amelioration of proteinuria and might not be attributed to a direct ACE inhibitor action. Since dyslipidemia may concur to propagate renal injury (33), the additive effects of ramipril and sitaxsentan in ameliorating the lipid profile in ZDF rats can be conceived as an additional factor of renal protection by the combined therapy.

Severe cardiac dysfunction in experimental and human diabetes may depend on massive cardiomyocyte remodeling and death, structural and functional alterations of the microcirculation (19, 24, 58). The myocardium of ZDF rats was likewise characterized by significant myocyte loss (21% of myocyte number), detrimental reactive hypertrophy (+28% myocyte volume), and capillary rarefaction (−24% capillary density) compared with lean rats. Hyperglycemia is a potent stimulus for ANG II, ET-1, and ET_a myocyte expression (13). A sudden increase in glucose levels is associated with myocyte apoptosis, responsible for the 30% myocyte loss in experimental type 1 diabetes (21). In the present study, failure of the single drugs as well as their combination to protect the diabetic heart from cardiomyocyte death might be due to the fact that treatments started too late to counteract the negative effect of hyperglycemia, which occurs in ZDF rats within 2 mo of age (4).

However, we cannot exclude that the persistence of cardiomyocyte loss in ZDF rats also depends on the disruption of myocyte turnover due to cardiac stem cell impairment (43). Finally, the possibility that myocardial ischemia could have contributed to LV cardiomyocyte loss has been ruled out by the observation of no changes in interstitial collagen in ZDF compared with lean rats, according to a previous study (23). An increase in cardiomyocyte volume, an adaptive response to high glucose-induced free radical generation which often leads to cardiac dysfunction in type 2 diabetes (53), was completely reversed in ZDF rats by the ACE inhibitor and ET_a receptor antagonist without increasing myocardial oxygen demand. The drug combination was twofold more effective in reducing myocyte volume compared with ramipril, which belongs to a drug class with a well-known antihypertrophic action on the diabetic myocardium (20).

Although to a lesser extent, sitaxsentan affected myocyte volume, in line with in vitro data showing the ability of ET-1 to induce cardiomyocyte hypertrophy through the ET_a receptor (14) and in vivo studies showing the efficacy of sitaxsentan in reducing dilation of the LV chamber in rat post-myocardial infarction (41).

Another observation is the reduced capillary density in the hearts of ZDF rats as previously reported in obese Zucker fatty rats (28), the original inbred strain of ZDF. Restoration of the capillary network provided by the ACE inhibitor plus ET_a receptor antagonist is an unprecedented finding for the heart microcirculation, and it is mainly sustained by the action of sitaxsentan. Myocardial expression of VEGF, a major mediator of neovascularization in the heart (59), was restored in ZDF rats to levels even higher than lean rats by sitaxsentan, in line with a previous study (31) and further enhanced by the drug combination. The effect of endothelin blockade on angiogenesis was reported a decade ago in a model of surgically induced hindlimb ischemia, in which a marked increase in vessel density was associated with activation of VEGF and endothelial nitric oxide synthase in ischemic legs after chronic ET antagonist treatment (30). The angiogenic response enhanced myocardial vascular density independently of myocyte number and volume enlargement. The “uncoupling” of the endothelial cell mass from the myocyte changes could be taken to suggest VEGF as the molecule responsible for preservation of cardiac structure through its binding to VEGF receptor-1, the expression of which is highly enhanced in the ZDF heart after sitaxsentan or the drug combination.

Extensive ultrastructural abnormalities in myocardium of ZDF rats on vehicle were attenuated by sitaxsentan and almost normalized by the drug combination. Protection of myocyte remodeling could be due to reduced peroxynitrite production by cardiomyocytes, resulting in less damaged mitochondria.

In conclusion, we provided evidence that concomitant blockade of ANG II synthesis and ET-1 biological activity through an ET_a receptor antagonist afforded renoprotection in advanced type 2 diabetes. The drug’s effect on the kidney was mainly due to an ACE inhibitor, leaving room for adding another compound targeting an additional pathogenetic pathway. Our work highlights a remarkable effect of the drug combination, mainly sustained by sitaxsentan, on cardiac structure and microvascular architecture through the action of VEGF-1.

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DISCLOSURES

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