Unlike each drug alone, lisinopril if combined with avosentan promotes regression of renal lesions in experimental diabetes

Elena Gagliardini,1* Daniela Corna,1* Carla Zoja,1 Fabio Sangalli,1 Fabiola Carrara,1 Matteo Rossi,1 Sara Conti,1 Daniela Rottoli,1 Lorena Longaretti,1 Andrea Remuzzi,1,2 Giuseppe Remuzzi,1,3 and Ariela Benigni1

1Mario Negri Institute for Pharmacological Research, 2Mechanical and Industrial Engineering Department, University of Bergamo, and 3Unit of Nephrology and Dialysis, Azienda Ospedaliera Ospedali Riuniti di Bergamo, Bergamo, Italy

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Gagliardini E, Corna D, Zoja C, Sangalli F, Carrara F, Rossi M, Conti S, Rottoli D, Longaretti L, Remuzzi A, Remuzzi G, Benigni A. Unlike each drug alone, lisinopril if combined with avosentan promotes regression of renal lesions in experimental diabetes. Am J Physiol Renal Physiol 297: F1448–F1456, 2009. First published August 12, 2009; doi:10.1152/ajprenal.00340.2009.—In the present study, we evaluated the effect of simultaneously blocking angiotensin II synthesis and endothelin (ET)-1 activity as a multimodal intervention to implement renoprotection in overt diabetic nephropathy. Mechanisms underlying combined therapy effectiveness were addressed by investigating podocyte structure and function and glomerular barrier size-selective properties. Uninephrectomized rats made diabetic by streptozotocin received orally placebo, lisinopril (12.5 mg/l), the ET_A receptor antagonist avosentan (30 mg/kg), or their combination from 4 (when animals had proteinuria) to 8 mo. Proteinuria, renal damage, podocyte number, nephrin expression, and glomerular size selectivity by graded-size Ficoll molecule fractional clearance were assessed. Combined therapy normalized proteinuria, provided complete protection from tubulointerstitial damage, and induced regression of glomerular lesions, while only a partial renoprotection was achieved by each drug alone. Lisinopril plus avosentan restored to normal values the number of podocytes. Single therapies only limited podocyte depletion. Defective nephrin expression of diabetes was prevented by each drug. Altered glomerular size selectivity to large macromolecules of diabetic rats was remarkably improved by lisinopril and the combined treatment. Avosentan ameliorated peritubular capillary architecture and reduced interstitial inflammation and fibrosis. The ACE inhibitor and ET_A receptor antagonist induced regression of glomerular lesions in overt diabetic nephropathy. Regression of renal disease was conceivably the result of the synergistic effect of the ACE inhibitor of preserving glomerular permselective properties and the ET_A antagonist in improving tubulointerstitial changes. These findings provide mechanistic insights to explain the antiproteinuric effect of this combined therapy in diabetes.

* E. Gagliardini and D. Corna contributed equally to this work.

Address for reprint requests and other correspondence: E. Gagliardini, Mario Negri Institute for Pharmacological Research, Via Gavazzeni, 11, 24125 Bergamo, Italy (e-mail: elena.gagliardini@marionegrri.it).

In patients with early renal involvement (37), lowering blood pressure and the levels of urinary albumin by agents that inhibit the renin angiotensin system (RAS) reduces the risk of end-stage kidney disease (ESKD), as well as that of myocardial infarction, heart failure, and stroke (15). Effectiveness of RAS inhibitors crucially depends on the time at which the treatment is started, to the extent that imperfect renoprotection was observed when therapy was given in the advanced phase of the disease (27). In this context, 10% of patients with overt proteinuria and worsening kidney function continue to progress to ESKD or die prematurely of myocardial infarction or stroke every year despite RAS inhibitor therapy (10, 20). Novel and multimodal intervention strategies targeting pathogenic pathways other than angiotensin II are therefore worth exploring for diabetic patients who remain at high risk of poor renal and cardiovascular outcomes.

Endothelin-1 is a central player in progressive renal injury by virtue of its vasoactive, profibrogenic, and inflammatory properties (28). The effects of ET-1 are mediated by two cell-surface receptors, the type A (ET_A) and the type B (ET_B) receptors. ET_A receptors reside in vascular smooth muscle cells and mediate vasoconstriction and cell proliferation, while ET_B receptors are present on endothelial cells and are mainly vasodilatory via prostacyclin and nitric oxide stimulation (28). Besides the vasodilatory effect, ET_B receptors act as clearance receptors for circulating ET-1 and regulate water and sodium handling (28). Renal synthesis of ET-1 is increased in experimental and human diabetes (4, 40), and endothelin receptor antagonists reduced renal dysfunction and prevented renal inflammation in experimental diabetes (4, 39).

An interference of the RAS on the ET system has been reported as indicated by angiotensin II-induced activation of renal ET synthesis (2), as well as by the effect of ACE inhibitors in reducing renal ET-1 formation in experimental progressive nephropathy (19, 30, 49). Data are also available that angiotensin II and ET-1 act on the kidney through independent pathways, as shown in mice lacking AT_1 receptor in which progression of kidney disease was dependent on excess renal synthesis of ET-1 (5). These findings offer the rationale for evaluating the effect of simultaneously interrupting angiotensin II synthesis and ET-1 activity to implement renoprotection in advanced diabetic nephropathy.

In this study, we investigated whether the renoprotective action of the combined therapy of ACE inhibitor plus ET_A antagonist translated into an amelioration of renal structure and function in rats with overt diabetic nephropathy. Specifically, we focused on the effect of the combined therapy on podocyte and glomerular barrier size-selective abnormal-

DIABETES MELLITUS IS A PUBLIC health concern, and projections for the future are alarming (46a). About one-third of diabetic patients have progressive deterioration of renal function and ultimately require renal replacement therapy by dialysis or transplantation (35). Of great concern, type 2 diabetic patients with renal involvement eventually die of cardiovascular disease threefold more than diabetic patients without signs of renal failure (1).
MATERNAL AND METHODS

Experimental design. Sprague-Dawley rats (Charles River Italia, Calco, Italy) with initial body weights of 260–300 g were used. Animal care and treatment were conducted in accordance with the institutional guidelines that are in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale suppli 40, 18 febbraio 1992. Circolare n.6, Gazzetta Ufficiale 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL358–1, December 1987; Guide for the Care and Use of Laboratory Animals, National Research Council, 1996). All animals were housed in a room in which the temperature was kept constant on a 12:12-h light-dark cycle and allowed free access to a standard diet and tap water. Seven days after a right nephrectomy was performed to accelerate the disease, diabetes was induced by an injection of streptozotocin (60 mg/kg iv; Sigma, St Louis, MO). The presence of diabetes was confirmed 2 days later by the measurement of the tail blood glucose level with a reflectance meter (ASCENSSIA ELITE, Bayer, Milan, Italy). Diabetic rats received a daily evening injection of insulin (Ultratard HM or Protaphane HM; Nordisk Farmaceutici, Rome, Italy), in doses individually adjusted to maintain blood glucose levels (Ultratard HM or Protaphane HM; Nordisk Farmaceutici, Rome, Italy) (14) (30 mg/kg by gavage) (42); and lisinopril (pyridine-4-yl)-pyrimidin-4-yl]-amide, Speedel Pharma, Basel, Switzerland)

Renal histology. The removed kidneys were fixed in Dubosque-Brazil and embedded in paraffin. Three-micrometer sections were stained with Masson’s trichrome, hematoxylin and eosin, or periodic acid-Schiff reagent. At least 100 glomeruli were examined for each animal, and the extent of glomerular damage was expressed as the percentage of glomeruli presenting sclerotic lesions. Tubular changes (atrophy, casts, and dilation) were graded from 0 to 4 (0, no changes; 1, changes affecting <25% of the sample; 2, changes affecting 25–50% of the sample; 3, changes affecting 50–75% of the sample; 4, changes affecting 75–100% of the sample). All renal biopsies were analyzed by the same pathologist, who was unaware of the nature of the experimental groups.

Immunohistochemical analysis. Indirect immunofluorescence was performed for the detection of interstitial macrophage infiltration (31). The alkaline phosphatase-Fast Red technique was used to evaluate transforming growth factor (TGF)-β expression (32). An immunoperoxidase method was employed for nephrin and type III collagen detection (7, 31). The following primary antibodies were used: mouse anti-ED1 antigen present in rat monocytes/macrophages (diluted 1:100, Chemicon International, Temecula, CA); rabbit anti-TGF-β (1:100, Santa Cruz Biotechnology, Santa Cruz, CA); goat anti-nephrin (1:100, Santa Cruz Biotechnology); and rabbit anti-type III collagen (1:100, Chemicon). ED1-positive cells were counted in 13 randomly selected high-power microscopic fields (×400) on average/animal. The intensity of TGF-β, nephrin, and type III collagen signals was graded on a scale of 0 to 3 (0, no staining; 1, weak staining; 2, staining of moderate intensity; 3, strong staining). Negative controls were obtained by omitting the primary antibody on adjacent sections.

Table 1. Effect of lisinopril and avosentan alone or in combination on body weight, food intake, serum cholesterol, serum triglycerides, and systolic blood pressure in diabetic rats at 8 mo

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Food Intake, g/day</th>
<th>Serum Cholesterol, mg/dl</th>
<th>Serum Triglycerides, mg/dl</th>
<th>Systolic Blood Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>472±12b</td>
<td>41±2b</td>
<td>104±3.2</td>
<td>123.68±13.60a</td>
<td>153±4b</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>482±16b</td>
<td>40±2b</td>
<td>103±2.5</td>
<td>116.46±11.39b</td>
<td>117±3d</td>
</tr>
<tr>
<td>Avosentan</td>
<td>488±10b</td>
<td>43±2b</td>
<td>104±3.8</td>
<td>97.10±9.84ac</td>
<td>145±2b</td>
</tr>
<tr>
<td>Lisinopril+avosentan</td>
<td>449±16b</td>
<td>34±1</td>
<td>100±0.1</td>
<td>108.30±5.33c</td>
<td>105±4ACE</td>
</tr>
<tr>
<td>Control</td>
<td>746±25</td>
<td>26±2</td>
<td>102±2.0</td>
<td>76.33±3.48</td>
<td>121±2</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, **P < 0.01 vs. control. †P < 0.05, ‡P < 0.01 vs. placebo. §P < 0.01 vs. avosentan.

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be equal to 45 mmHg in normal controls, 55 mmHg in diabetic rats given placebo, 45 mmHg in rats on lisinopril and combined treatment, and 50 mmHg in animals receiving avosentan.

Statistical analysis. Results are expressed as means ± SE. Data of body weight, food intake, water intake, diuresis, systolic blood pressure, and mRNA expression were analyzed using ANOVA with the Bonferroni post hoc analysis for multiple comparisons. Fractional clearance data were log-transformed before ANOVA. The nonparametric Kruskal-Wallis test for multiple comparisons was used to analyze data of serum cholesterol, triglycerides, ALT, AST, proteinuria, albuminuria, renal histology, immunohistochemistry, and morphometric parameters. The statistical significance level was defined as \( P < 0.05 \).

RESULTS

Mortality and systemic parameters. By the end of the study, the following mortality was recorded: 1 of 10 rats in the placebo group, 4 of 17 rats in the lisinopril group (1 of 4 was euthanized because of a testis tumor), 5 of 18 rats in the avosentan group, and 3 of 10 rats in the combined therapy group.

Diabetic rats, independently of the treatment, gained weight during the study although to a lesser extent than controls (Table 1). Food intake was not different among groups of diabetic rats during the study period. Serum cholesterol levels were comparable among the groups of diabetic and control rats. Triglyceride levels were significantly increased in diabetic rats receiving placebo with respect to controls. Hypertriglyceridemia was significantly reduced by avosentan and combined therapy with respect to placebo (Table 1). Avosentan-treated rats did not show signs of hepatic toxicity as documented by serum ALT and AST levels (ALT: 52.8 ± 5.9; AST: 88.7 ± 13.3 U/l), which were comparable to those of rats given placebo (ALT: 62.4 ± 7.2; AST: 90.9 ± 13.1 U/l).

At randomization (4 mo), diabetic rats had a significant increase in SBP with respect to controls (137.3 ± 0.9 vs. 103 ± 4 mmHg, \( P < 0.01 \)). SBP further increased over time in diabetic rats given placebo (Table 1). A significant (\( P < 0.01 \)) reduction of SBP levels was observed after lisinopril alone or in combination with avosentan with respect to placebo at 8 mo.

![Fig. 1](http://ajprenal.physiology.org/)

**Fig. 1.** Urinary protein (A) and albumin (B) excretion levels measured in control and diabetic rats at 4 mo (before treatment) and 8 mo in control and diabetic rats given placebo, lisinopril, avosentan, and lisinopril + avosentan. Values are means ± SE. \( *P < 0.05 \), \( **P < 0.01 \) vs. control. \( \#P < 0.01 \) vs. placebo. \( \#\#P < 0.01 \) vs. avosentan. \( SP < 0.05 \) vs. lisinopril at corresponding time points.

![Fig. 2](http://ajprenal.physiology.org/)

**Fig. 2.** Incidence of glomerulosclerosis in diabetic animals at 4 mo (before treatment) and at 8 mo after placebo, lisinopril, avosentan, or combined therapy and in control rats. Values are means ± SE. \( *P < 0.05 \), \( **P < 0.01 \) vs. control. \( \#P < 0.05 \), \( \#\#P < 0.01 \) vs. 4 mo. \( \#\#\#P < 0.01 \) vs. placebo at 8 mo. \( \#\#\#P < 0.01 \) vs. monotherapies.

CTTCAAGGACGGTCTGTATTG -3', reverse 5'-AATTGGGCTCTCCGTGATTC-3'; and GAPDH forward 5'-TCATCCCTGCATCCTGCTG-3', reverse 5'-CTGGGATGACCTTGCCCAC-3'. The \( \Delta \Delta C_t \) technique was used to calculate cDNA content in each sample using the cDNA expression in control rats as a calibrator.

Renal hemodynamics and glomerular permeability to macromolecules. Whole kidney glomerular filtration rate (GFR) and renal plasma flow (RPF) were evaluated as previously described (12). The size-selectivity properties of the glomerular capillary wall were examined by measuring the fractional clearance of neutral graded-size FITC-Ficoll molecules as previously described (12). Theoretical analysis of fractional clearance data was performed using the heteroporous model previously described (34), assuming the lognormal plus shunt distribution of membrane pores (13). The transmembrane pressure difference (\( \Delta P \)) was assumed on the basis of micropuncture data reported in literature for diabetic animals (47, 48). In detail, \( \Delta P \) was assumed to be equal to 45 mmHg in normal controls, 55 mmHg in diabetic rats given placebo, 45 mmHg in rats on lisinopril and combined treatment, and 50 mmHg in animals receiving avosentan.
Table 2. Effect of lisinopril and avosentan alone or in combination on tubular damage, inflammation, and fibrosis in diabetic rats at 8 mo

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubular Damage Score</th>
<th>ED1⁺ Cells, cells/HPF</th>
<th>TGF-β Score</th>
<th>Collagen III Score</th>
<th>MMP-9 mRNA, relative expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>1.11 ± 0.2a</td>
<td>14.02 ± 8.96b</td>
<td>2.44 ± 0.13b</td>
<td>2.39 ± 0.14b</td>
<td>0.34 ± 0.08</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>0.31 ± 0.13c</td>
<td>5.04 ± 2.17c</td>
<td>1.15 ± 0.09d</td>
<td>1.23 ± 0.11d</td>
<td>1.59 ± 0.26c</td>
</tr>
<tr>
<td>Avosentan</td>
<td>0.38 ± 0.14c</td>
<td>5.23 ± 3.55c</td>
<td>1.50 ± 0.14d</td>
<td>1.35 ± 0.13c</td>
<td>1.45 ± 0.08d</td>
</tr>
<tr>
<td>Lisinopril + avosentan</td>
<td>0.14 ± 0.14d</td>
<td>3.10 ± 1.15c-e</td>
<td>0.93 ± 0.07e</td>
<td>1.0 ± 0.1d</td>
<td>1.64 ± 0.21d</td>
</tr>
<tr>
<td>Control</td>
<td>0.17 ± 0.17</td>
<td>3.63 ± 1.40</td>
<td>0.83 ± 0.17</td>
<td>1.0 ± 0.1</td>
<td>1.04 ± 0.13</td>
</tr>
</tbody>
</table>

Values are means ± SE. HPF, high-power field (×400). *P < 0.05, **P < 0.01 vs. control. **P < 0.05, ***P < 0.01 vs. placebo. *P < 0.01 vs. avosentan.

(Table 1). In the group of rats receiving avosentan, SBP levels were only numerically lower.

**Urinary protein and albumin excretion.** As shown in Fig. 1A, at 4 mo rats with diabetes exhibited significantly higher values (P < 0.01) of urinary protein excretion than controls. Proteinuric increased in rats given placebo, averaging 152 ± 31 mg/day at 8 mo. Lisinopril maintained proteinuria to levels fairly comparable to those measured at 4 mo (52 ± 4 mg/day), with a 66% reduction with respect to the placebo group. Mean values of proteinuria of rats treated with avosentan were numerically lower than those of rats given placebo, with a percentage reduction of 32%. The combination of lisinopril and avosentan further reduced proteinuria to values (35 ± 5 mg/day) that were significantly different (P < 0.01) from those of both placebo and single therapies, and comparable to control rats (31 ± 2 mg/day). A similar trend was observed for urinary albumin (Fig. 1B).

**Renal histology.** Morphological evaluation of the kidney in diabetic rats at 4 mo revealed the presence of 1.50 ± 0.22% on average of sclerotic glomeruli (P < 0.05 vs. the age-matched controls: 0.08 ± 0.003%). At 8 mo, diabetic rats given placebo showed variable degrees of glomerulosclerosis and hyalinosis with segmental collapse of the glomerular tuft affecting a significantly higher percentage of glomeruli with respect to diabetic rats at 4 mo (P < 0.05) and controls (Fig. 2). The incidence of glomerulosclerosis in rats given lisinopril was significantly lower than that found in the placebo group. Avosentan also decreased the percentage of glomerular sclerosis with respect to placebo. The combined therapy normalized glomerular histology. Of note, the percentage of sclerotic glomeruli observed in animals receiving lisinopril plus avosentan (0.14 ± 0.14%) was significantly lower (P < 0.01) than that observed in diabetic animals at 4 mo, suggesting regression of glomerular lesions (Fig. 2).

In diabetic rats, glomerular injury was associated with a mild tubular damage, which progressively increased from 4 (score: 0.50 ± 0.22, P < 0.05 vs. age-matched controls: 0.079 ± 0.002) to 8 mo (score: 1.11 ± 0.2, Table 2). Tubular damage was reduced by either lisinopril or avosentan, and normalized by the combined therapy (Table 2).

**Fibrosis and inflammation markers.** Glomerulosclerosis in untreated diabetic animals at 8 mo was associated with a decreased expression of MMP-9, the enzyme principally involved in matrix degradation (22), with respect to controls (Table 2). ACE inhibitor, ETA receptor antagonist, or their combination significantly increased the mRNA levels of MMP-9, thereby suggesting an activation of matrix reabsorption induced by the drugs.

Accumulation of ED1⁺ monocytes/macrophages was found in peritubular cortical interstitium of diabetic animals receiving placebo (Table 2). Lisinopril, avosentan, and to a greater extent the combined therapy significantly reduced the interstitial accumulation of inflammatory cells. Tubulointerstitial abnormalities observed in diabetic rats were associated with a significant increase in tubular expression of the profibrotic factor TGF-β and interstitial accumulation of type III collagen with respect to controls (Table 2). Both lisinopril and avosentan significantly limited tubular TGF-β staining and interstitial collagen deposition, which were comparable to controls in the combined therapy group.

**Peritubular capillary structure.** Alterations in peritubular capillary structure were observed in diabetic rats given placebo with respect to controls, as demonstrated by the reduction in length density of peritubular capillaries (LV), volume density of capillary lumens (Vv), and capillary diameter (Table 3). Lisinopril increased numerically LV, and the mean diameter of capillaries and significantly Vv. Avosentan had no effect on LV, but increased numerically Vv and significantly the mean diameter of capillaries. All parameters were normalized after combined therapy (Table 3).

**Podocyte number and nephrin expression.** Glomerular hypertrophy was observed in diabetic rats on placebo. Treat-

Table 3. Peritubular capillary (PC) and podocyte morphometric determinations in diabetic rats at 8 mo

<table>
<thead>
<tr>
<th>Group</th>
<th>PC Length Density (LV), mm/m²</th>
<th>PC Volume Density (Vv), mm³/mm³</th>
<th>PC Mean Diameter, μm</th>
<th>Mean Glomerular Volume, 10⁶ μm³</th>
<th>Podocyte Number/Volume, 1/10⁶ μm²</th>
<th>Podocyte Number/ Glomerulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>1.082 ± 0.27</td>
<td>0.061 ± 0.004</td>
<td>8.4 ± 0.22</td>
<td>2.82 ± 0.18³</td>
<td>64.64 ± 3.04b</td>
<td>181.56 ± 5.60b</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>1.217 ± 0.92</td>
<td>0.079 ± 0.003³</td>
<td>9.1 ± 0.34</td>
<td>2.45 ± 0.12²</td>
<td>81.58 ± 3.78b³</td>
<td>196.90 ± 7.64</td>
</tr>
<tr>
<td>Avosentan</td>
<td>1.035 ± 0.44</td>
<td>0.079 ± 0.006³</td>
<td>9.9 ± 0.51³</td>
<td>2.55 ± 0.13³</td>
<td>79.44 ± 5.45³c</td>
<td>197.30 ± 9.00</td>
</tr>
<tr>
<td>Lisinopril + avosentan</td>
<td>1.150 ± 0.88</td>
<td>0.101 ± 0.005±c</td>
<td>10.6 ± 0.56³-e</td>
<td>2.34 ± 0.07a</td>
<td>93.34 ± 2.76³d</td>
<td>218.00 ± 6.5³</td>
</tr>
<tr>
<td>Control</td>
<td>1.157 ± 0.17</td>
<td>0.083 ± 0.009</td>
<td>9.6 ± 0.55</td>
<td>1.96 ± 0.10</td>
<td>112.97 ± 4.99</td>
<td>219.83 ± 9.71</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, **P < 0.01 vs. control; **P < 0.05, ***P < 0.01 vs. placebo. *P < 0.05 vs. lisinopril. *P < 0.05 vs. avosentan.
ments only numerically reduced the glomerular volume (Table 3).

The podocyte volume density was significantly reduced in diabetic rats given placebo with respect to controls \( (P < 0.01) \). Lisinopril and avosentan, and to a greater extent the combined therapy, limited the decrease in podocyte volume density (Table 3). The number of podocytes per glomerulus was significantly decreased in placebo-diabetic rats \( (P < 0.05\) vs. controls) and restored to normal values by the combined therapy (Table 3).

An intense expression signal of nephrin protein with the typical epithelial-like staining pattern was detected in glomeruli of control animals (score: 2.5 ± 0.14, Fig. 3E). Diabetic rats given placebo exhibited a significant \( (P < 0.01) \) reduction in

![Fig. 3. Representative photomicrographs of nephrin expression by immunoperoxidase in diabetic animals receiving placebo (A), lisinopril (B), avosentan (C), or combined therapy (D) and in control rats (E). Staining was completely abrogated omitting the primary antibody on the adjacent section on each slide, indicating staining specificity (F). Original magnification, ×1,000.](image-url)
neprin protein expression (score: 1.46 ± 0.12, Fig. 3A) compared with controls. Neprin expression was normalized by lisinopril (score: 2.58 ± 0.07, Fig. 3B) and avosentan (score: 2.52 ± 0.1, Fig. 3C) given alone or in combination (score: 2.59 ± 0.06, Fig. 3D).

**Renal hemodynamics and glomerular size selectivity.** In placebo-treated diabetic rats, GFR and RPF were numerically lower than controls (Table 4). In rats given lisinopril, both GFR and RPF increased, although a statistical difference was not achieved. Avosentan did not affect GFR values, while it tended to increase RPF. The combined therapy favorably affected RPF (Table 4).

The size-selective properties of the glomerular capillary barrier were assessed by the fractional clearance of graded-size FITC-Ficoll molecules (molecular radii ranging from 20 to 74 Å). In placebo-treated diabetic animals, fractional clearance of the largest molecules was higher than in controls (Fig. 4A). As expected, lisinopril reduced the fractional clearance of the largest Ficoll molecules (Fig. 4B). In diabetic rats treated with avosentan, fractional clearances of the largest test molecules was even higher than in the placebo group (Fig. 4C). The combined therapy showed results comparable to those obtained with lisinopril alone (Fig. 4D).

Data on Ficoll fractional clearance and renal hemodynamics were used to calculate intrinsic glomerular membrane permeability properties using the lognormal probability distribution of pore size. The glomerular ultrafiltration coefficient ($K_f$) was significantly lower ($P < 0.05$) in diabetic animals than in controls (Table 4). In rats receiving lisinopril or the combined therapy, $K_f$ was normalized. On the contrary, avosentan alone did not affect significantly $K_f$. No statistically significant differences were observed in mean ($\mu$) and standard deviations ($\sigma$) of pore size (Table 4). The shunt parameter ($\omega_0$), which represents the fraction of filtrate volume passing through the nonselective pores, was higher in diabetic animals given placebo compared with controls (Fig. 4). Lisinopril and the combination therapy normalized $\omega_0$. Avosentan alone did not affect $\omega_0$.

**DISCUSSION**

The present results show that combined treatment of a ACE inhibitor and ETA receptor antagonist fully eradicated proteinuria and provided complete protection from glomerular and tubulointerstitial injury in diabetic rats with overt nephropathy. Lisinopril and avosentan given alone were only partially renoprotective. Of note, combined therapy reversed glomerular lesions to the extent that the percentage of glomeruli affected by sclerosis in treated rats was even lower than that measured before the start of treatment. Regression of renal lesions, previously described with bosentan alone in hypertensive rats (9), is an unprecedented finding for the ACE inhibitor and ETA receptor antagonist combination in diabetes. Finding that the combined treatment fully normalized proteinuria prompted us to focus subsequent studies on the evaluation of podocyte structure and function and glomerular size-selective properties. The integrity of podocytes, which span the capillaries and stabilize the whole glomerular barrier, is crucial for glomerular function. In diabetes, progression of the disease is associated with progressive loss of podocytes. Podocyte depletion induces the remaining podocytes to cover the denuded glomerular basement membrane, but further compensation for cell loss leads to the expansion of the mesangial compartment (41) with the possible net result of glomerular hypertrophy in diabetes. In the present study, morphometric analysis confirmed the loss of podocytes per glomerulus in diabetic rats receiving placebo, which paralleled the development of glomerulosclerosis. Lisinopril and avosentan limited podocyte depletion without leading to a complete normalization (16), which was instead achieved by the combined therapy. The mechanism behind the protective effect on podocyte number is unknown. The mature podocyte has limited capacity to divide (46). Possible mechanisms for podocyte replacement are represented by the contribution of bone marrow stem cells that act as a reservoir for glomerular cells (17, 29) or the migration of progenitor cells recently identified in Bowman's capsule which could acquire a podocyte phenotype (38). Which of the two options is valuable in the present setting is worth investigating, but goes beyond the scope of this study.

Structural changes in podocytes were coupled with molecular alterations documented by the expression of nephrin, the slit diaphragm protein which functions to maintain slit pore integrity and renal filtration capacity (43). We found that the reduction of nephrin protein expression in the glomeruli of diabetic rats was prevented by the ACE inhibitor, consistent with previous observations in experimental and human diabetic nephropathy (8, 18). A novel finding here was the demonstration that the ETA receptor antagonist also normalized the defective nephrin levels in diabetes. This could have been the consequence of the effect of avosentan of limiting podocyte loss.

We then investigated whether the remarkable effect of drug combination in restoring podocyte integrity and nephrin expression was accompanied by changes in glomerular permselective function. The mathematical model reported before (34) allowed us to derive hemodynamic parameters. Fractional clearance of graded-size Ficoll molecules with a globular configuration similar to plasma proteins (25) was instrumental in assessing the nature of size-selective function of the glo-

**Table 4. Total kidney GFR and RPF and calculated membrane permeability parameters in diabetic rats at 8 mo**

<table>
<thead>
<tr>
<th>Group</th>
<th>GFR, ml·min⁻¹·100 g⁻¹</th>
<th>RPF, ml·min⁻¹·100 g⁻¹</th>
<th>$K_f$, ml·min⁻¹·mmHg⁻¹</th>
<th>$\mu$, Å</th>
<th>$\sigma$, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Placebo</td>
<td>0.42±0.05</td>
<td>1.17±0.09</td>
<td>0.113±0.016a</td>
<td>31.3±0.7</td>
<td>1.238±0.006</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>0.53±0.07</td>
<td>1.63±0.23</td>
<td>0.202±0.014a</td>
<td>28.4±0.5</td>
<td>1.259±0.006</td>
</tr>
<tr>
<td>Avosentan</td>
<td>0.39±0.06</td>
<td>1.30±0.13</td>
<td>0.090±0.002b</td>
<td>32.2±0.2</td>
<td>1.238±0.002</td>
</tr>
<tr>
<td>Lisinopril+avosentan</td>
<td>0.41±0.04</td>
<td>1.53±0.13</td>
<td>0.229±0.015c</td>
<td>29.5±0.7</td>
<td>1.245±0.008</td>
</tr>
<tr>
<td>Control</td>
<td>0.46±0.07</td>
<td>1.34±0.20</td>
<td>0.246±0.010</td>
<td>33.4±0.6</td>
<td>1.210±0.006</td>
</tr>
</tbody>
</table>

Values are means ± SE. GFR, glomerular filtration rate; RPF, renal plasma flow; $K_f$, glomerular ultrafiltration coefficient; $\mu$, mean pore size of lognormal pore distribution; $\sigma$, SD of lognormal pore distribution; *P < 0.05, **P < 0.01 vs. control. ***P < 0.05 vs. placebo. ****P < 0.05 vs. avosentan.
Fig. 4. Fractional clearance of graded-size Ficoll molecules evaluated at 8 mo in controls and diabetic rats treated with placebo, lisinopril, avosentan, and combined therapy (A–D, respectively). Inset: histograms show the shunt parameter values (ω0) of the different experimental groups.
merular capillary wall. In line with previous studies (33, 36), late-stage diabetes was associated with increased clearance of large Ficoll molecules (>55 Å), suggesting that alterations in the glomerular barrier were mainly confined to large nonelective pores responsible for the passage of circulating macromolecules into the urinary space. Lisinopril ameliorated the glomerular membrane size-selective dysfunction, which translated into an antiproteinuric effect, albeit not complete. Blockade of ET-1 activity by avosentan did not affect the mean (u) and the membrane pore radii distribution (s) and the nonelective shunt pathway (ωh). Despite the presence of large pores perforating the glomerular basement membrane, avosentan partially lowered proteinuria and albuminuria. This could be due to the effect of the ETA receptor antagonist on renal hemodynamics as documented by the tendency of renal blood flow to ameliorate in avosentan-treated animals. In addition, avosentan, which increased peritubular capillary perfusion, could have ameliorated tubular function, resulting in more protein reabsorption. Adding the ETA receptor antagonist to the ACE inhibitor reduced nonelective pore dimensions to normal values, in harmony with findings of normalization of proteinuria. Combined therapy increased volume density, diameter and, although numerically, the length of peritubular capillaries, all of which were invariably reduced in diabetes. This effect could have been the consequence of amelioration of renal hemodynamics. However, a recent study showed in hypercholesterolemic pigs that a ETA receptor antagonist improved renal microvascular architecture without changes in renal hemodynamics (11). The restored peritubular vascularization after combined therapy could have contributed to the reduction of tubular lesions and interstitial damage as indicated by reduced interstitial inflammation and decreased expression of fibrosis markers in the kidney of diabetic animals receiving lisinopril plus avosentan. These findings tend to suggest that the effect of the drug combination of fully protecting animals from renal injury is the result of two diverse mechanisms acting synergistically. The ACE inhibitor preserved the glomerular permselective properties while the ET antagonist improved the architecture of peritubular capillaries and, therefore, renal interstitial blood perfusion, and preserved tubulointerstitial structural integrity, which would possibly lead to amelioration of tubular function and protein reabsorption.

The beneficial effect of the drug combination was apparently independent from the blood pressure reduction, as revealed by the fact that no significant difference in blood pressure levels was reached in lisinopril plus avosentan- vs. lisinopril-treated animals. Whether a more precise blood pressure measurement, as by telemetry, could have pointed out a difference cannot be ruled out. However, the lack of a significant additive blood pressure-lowering effect of avosentan on top of the ACE inhibitor is in line with the recent clinical data by Wenzel et al. (45).

An unexplained finding of this study is the increased mortality recorded in treated diabetic animals, which is unlikely attributable to hepatic toxicity of avosentan therapy. Future ad hoc studies will be devoted to evaluate possible cardiac side effects.

In conclusion, our results indicate that combined administration of ACE inhibitor and ETA receptor antagonist in overt diabetic nephropathy induced regression of renal lesions, preserved podocyte integrity and function, and normalized the altered size selectivity of the glomerular barrier. These findings provide mechanistic insights into previous clinical observations (45) and explain the antiproteinuric effect of such a combined therapy in diabetes.

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GRANTS

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