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Long-Term Effects of the Endothelin$_A$ Receptor Antagonist LU 135252 and the Angiotensin-Converting Enzyme Inhibitor Trandolapril on Diabetic Angiopathy and Nephropathy in a Chronic Type I Diabetes Mellitus Rat Model

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ABSTRACT

Diabetic angiopathy is a serious problem in antidiabetic therapy. We wanted to investigate whether treatment with the endothelin$_A$ receptor antagonist LU 135252 or with the angiotensin-converting enzyme inhibitor trandolapril might prevent angiopathy in long-term type I diabetes mellitus. Six groups of male Wistar rats were investigated: untreated age-matched control rats, healthy controls treated with trandolapril (0.3 mg/kg), healthy controls treated with LU 135252 (100 mg/kg), untreated diabetic rats, and diabetic rats treated with either trandolapril or LU 135252. Rats were rendered diabetic by injection of streptozotozin. Duration of the disease was 6 months. Thereafter, rats were sacrificed, and hearts, kidneys, and a mesenterial loop were removed. Hearts and kidneys were processed histologically; the mesenterial loop was perfused with saline at constant pressure for investigation of microvessels using microvideoangiometry while treated with either 30 mM KCl, 1 μM acetylcholine, or 1 μM sodium nitroprusside. All diabetic rats developed hyperglycemia without differences among these three groups. Diabetic rats exhibited marked anemia, which was significantly antagonized by both treatments. The heart capillaries/muscle fibers ratio was decreased significantly in diabetic animals, which was prevented fully by both treatments. Renal glomerular diameter was increased in diabetic rats. This was significantly antagonized by LU 135252 but not by trandolapril. Deposition of homogeneous eosinophilic material within the glomeruli was nearly completely prevented by LU 135252. The acetylcholine-induced vasodilation in mesenteric microvessels was significantly attenuated in diabetic rats, which was significantly antagonized by both treatments. We conclude that both angiotensin and endothelin seem to contribute to the development of diabetic angiopathy and that, in addition to angiotensin-converting enzyme inhibition, blockade of endothelin$_A$ receptors may be an interesting new approach to antiangiopathic therapy.

Diabetic angiopathy and nephropathy are still among the most serious chronic problems encountered in antidiabetic therapy. In previous studies, an involvement of the renin-angiotensin system has been demonstrated by several authors, and antiangiopathic effects of angiotensin-converting enzyme (ACE) inhibition were also found (Lewis et al., 1993; Olbrich et al., 1996; Rösen et al., 1996; O'Discroll et al., 1997). Diabetic angiopathy is associated with endothelial dysfunction leading to impaired nitric oxide (NO) release and, thus, to altered regulation of vascular tone (Olbrich et al., 1996, 1999). In addition to angiotensin II, enhanced endothelin (ET) plasma concentrations have been suggested to participate in the pathophysiology of diabetic angiopathy (Nakamura et al., 1995; Moreau et al., 1997; Mangiafica et al., 1998; Neri et al., 1998), and synergistic interaction between ET and the renin-angiotensin system has been postulated (Cameron and Cotter, 1996).

Thus, antagonization of either the angiotensin or ET pathway may exert antiangiopathic effects in diabetes mellitus. Inhibition of the angiotensin pathway using ACE inhibitors has been shown to improve vascular function in type I diabetic patients (O'Discroll et al., 1997). Regarding the ET pathway, ET can be released by many factors, including angiotensin II (Masaki and Yanagisawa, 1992), and acts via ET_A or ET_B receptors. Whereas (among other effects) endothelial ET_B receptors mediate NO release, ET_A receptors are involved in vasoconstrictive and proliferative effects of ET (Ohlstein et al., 1992; Simonson, 1994). In addition to angiotensin II, ET can promote cellular growth (Kobayashi et al., 1996), an effect dependent on a previous stimulation with

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ABBREVIATIONS: ACE, angiotensin-converting enzyme; NO, nitric oxide; ET, endothelin; SNP, sodium nitroprusside; ACh, acetylcholine; HDL, high density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GT, glutamyl transferase.

platelet-derived growth factor initiating mitosis. ET_A receptors are supposed to participate in this proliferation-promoting effect (Ohlstein et al., 1992). Protective effects of ET_A blockers have been demonstrated in various experimental models, e.g., in models of cardiac ischemic injury (Gonon et al., 1998; Raschack et al., 1998) or experimental heart failure (Mulder et al., 1998), in chronic transplant nephropathy or nephrectomy models (Orth et al., 1997, 1999), and in atherosclerosis (Kowala, 1997; Barton et al., 1998) and neointima formation in collared carotid arteries (Raschack et al., 1997) as well as in the reversal of angiotensin II-induced vascular hypertrophy (Moreau et al., 1997).

Thus, one could imagine that antagonization of ET_A receptors may exert positive effects in diabetic angiopathy and nephropathy. Because of the suggested pathophysiological role of ET in the development of diabetic angiopathy, several authors have used ET antagonists in various models of diabetes mellitus. Beneficial effects have been described with the unselective ET antagonist bosentan in a 6-week model of diabetic neuropathy (Stevens and Tomlinson, 1995) or PD142,893 in a model of diabetic proteinuria (Benigni et al., 1998). However, in the latter study, the drug was given after the onset of proteinuria and, thus, not as a prophylactic treatment. Selective blockade of ET_A receptors using either the peptide ET_A antagonist BQ123 in a 6-week model of diabetes mellitus investigating early nephropathy and vasculopathy (Cameron et al., 1994) or FR139317 in a 6-month model focusing on diabetic nephropathy (Nakamura et al., 1995) also have shown protective effects. However, it is unknown whether diabetes-induced endothelial dysfunction, cardiac capillary rarefication, cataracts, or anemia can be influenced by long-term ET_A blockade.

To evaluate the protective potential of ET_A blockade in diabetes mellitus, it is necessary to take both the generalized and chronic character of the disease into account. Thus, not only nephropathy as investigated in the other studies (mentioned above) but also endothelial dysfunction and angiopathy as well as typical late complications such as cataract development and anemia have to be considered. Moreover, the duration of the disease has to be long enough to allow full development of the typical changes observed in patients after a long duration of diabetes mellitus. To allow an evaluation of the effectiveness of ET_A blockade, a comparison with a treatment known to be effective, such as ACE inhibition, has to be included. In these respects, none of the previous studies was complete or long enough.

Thus, this study was undertaken to elucidate whether treatment with the new ET_A receptor antagonist LU 135252 or with an ACE inhibitor (included as the "golden standard" in this study) might be effective in preventing the typical broad spectrum of diabetic late complications, including angiopathy, anemia, cataracts, nephropathy, and endothelial dysfunction, found in patients in a chronic 6-month diabetes mellitus model (thus long enough to allow the development of all these diabetic complications). To our knowledge, this is the first study on the effects of an ET_A receptor antagonist (LU 135252) on the typical diabetic complications, including nephropathy, cataracts, blood parameters, angiopathy, and especially endothelial dysfunction, as well as cardiac and renal histology in the course of long-term type I diabetes mellitus in comparison with the effects of ACE inhibition.

Materials and Methods

All experiments were performed in accordance with the ethical rules of the Council for International Organization of Medical Science and the German laws for animal welfare. The experiments were approved by the local ethical committee. Six groups of male Wistar rats were investigated: untreated age-matched control rats ($n = 10$), healthy control rats treated with trandolapril (0.3 mg/kg b.wt.) ($n = 10$), healthy control rats treated with LU 135252 (100 mg/kg b.wt.) ($n = 9$) versus untreated diabetic rats ($n = 12$), diabetic rats treated with trandolapril ($n = 8$), and diabetic rats treated with LU 135252 ($n = 10$). The administered oral doses of trandolapril (Jouquey et al., 1994) and LU 135252 (Münter et al., 1996; Raschack et al., 1997) had been proven to be pharmacodynamically active in previous rat and rabbit experiments. The substances were administered by food mixture.

Rats (6 weeks old) were rendered diabetic by injection of streptozotozin (60 mg/kg) in the caudal vein. This led to the development of type I diabetes mellitus that was confirmed after a few days. One week after streptozotocin injection, drug treatment was started. Duration of the disease was 6 months. During this time, the cholesterol, high density lipoprotein (HDL), plasma glucose, blood pressure, heart rate, and kidney function were controlled (see Tables 1, 2, 3, and 4). After 6 months, the animals were sacrificed, heart and kidney were investigated histologically, and mesenteric artery function was tested.

Plasma Clinical Chemistry. The blood was sampled by retroorbital bleeding under short-term ether anesthesia. After centrifugation at 600*g* for 10 min, the plasma was collected and subjected to biochemical analysis. All clinical chemistry values were determined using a Hitachi 717 automated analyzer (Tokyo, Japan). Blood glucose was measured by the hexokinase method. Cholesterol and HDL (after precipitation) were measured enzymatically by the cholesterol oxidase/*p*-aminophenazone method. Triglycerides were determined also enzymatically. The liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transferase (γ-GT) were measured with a kinetic test. The urea was determined by a kinetic UV test. The creatinine was measured by the classical method of Jaffé.

Blood Pressure and Heart Rate. According to the method of Gerold and Tschirky (1968) systolic blood pressure was measured by noninvasive tail cuff plethysmography using a piezo sensor (BP recorder 8006; Ugo Basile, Varese, Italy) that also delivered the pulse rate by means of an inbuilt electronic counter.

Kidney Function. The urine was measured in 24-h collections in metabolic cages. Protein concentrations were measured with a pyrogallol red-molybdate complex reagent determined by a Hitachi 717 automated analyzer. The urine creatinine was measured (Jaffé method) to calculate the endogenous creatinine clearance.

Morphological Analysis. After 6 months, rats were stunned by a sharp blow on the neck and sacrificed rapidly by subsequent exsanguination. Hearts, kidneys, and a mesenterial loop were removed. Hearts and kidneys were fixed in formalin (40.5 ml of 35% formaldehyde, 5 ml of acetic acid, and distilled water to a final volume of 100 ml), dehydrated in isopropranolol, and embedded in paraffin after standard histological procedures. Slices of 6-μm thickness were prepared and stained with hematoxylin and eosin. The resulting slices of either kidney or heart were investigated using a Zeiss Axolab microscope (Zeiss, Köln, Germany) equipped with a Nikon F3 photocamera and digital image analysis system [frame grabber board: quick capture board (Data Translation, Marlboro, MA) and JAVA software (Jandel Scientific, Erkrath, Germany)]. For evaluation, the microscopic slides were blinded so that the investigator did not know to which group the actual preparation belonged. The following parameters were evaluated:

In the kidney the diameter of the glomeruli was measured at 1000× magnification. For each kidney, 30 glomeruli were investigated. In addition, the free width between the capillary tufts and

Bowman's capsule was measured (30 glomeruli per kidney). Furthermore, the deposition of homogeneous eosinophilic material, named "hyaline", in the glomeruli was examined. For quantification of this deposition, we used a three step scale: 0 = no hyaline present, 1 = one to two depositions, and 2 = more than two depositions.

In the heart, the number of capillaries and the number of cardiomyocytes in a given section of the left ventricle in which the fibers were transversely cross sectioned were evaluated at 1000× magnification so that the capillaries/muscle fiber ratio could be determined. In addition, the diameter of the muscle fibers was measured. We investigated 50 capillaries with the appertaining muscle fibers in each heart.

Vascular Function. For functional measurements of smooth muscle and endothelial function, a mesenteric loop was isolated with the appertaining intestine (length: 8 cm) according to the technique described earlier (Dhein et al., 1992; Olbrich et al., 1996). The mesenteric artery was cannulated and perfused with oxygenated Tyrode's solution (161.02 mM Na^+, 5.36 mM K^+, 1.8 mM Ca^{2+}, 1.05 mM Mg^{2+}, 146.86 mM Cl^-, 23.80 mM HCO_3^-, 0.42 mM $H_2PO_4^-$, 10.00 mM glucose , pH adjusted to 7.4 and gassed with 95% O_2 and 5% CO_2). An 8-cm loop of the small intestine was ligated, and all side branches of the mesenteric vessels were sealed by ligation so that an isolated mesenteric fold with the appertaining intestine and the perfusing arterial network was prepared. This preparation was fixed to a perfusion system with a constant perfusion pressure of 70 cm of H_2O, which corresponds to the actual physiological perfusion pressure in the mesenteric artery in this model. Ten cannulas were inserted into the intestine to provide drainage. With the help of a microscope (Zeiss) and a video camera (Sony, Tokyo, Japan), which was mounted behind the ocular of the microscope, the mesenteric vessels were displayed on a monitor (Sony). The total magnification was 240×. In the course of the experiments, pictures of the arteries were recorded. During the experiment, vessel diameters were determined directly on the screen and after the experiments were reevaluated in the digitalized pictures using a frame grabber board (Data Translation) with JAVA software (Jandel Scientific). Vessel diameter was assessed by analyzing the first derivative of the gray level along a cross sectional line (orthogonal to the vessel's longitudinal axis). The distance between the extremata corresponds to the vascular diameter. According to the generation theory of Ley and colleagues (Ley et al., 1986), we classified microvessels as G1 vessels, which are the branches perfusing the isolated loop. These vessels exhibited a diameter of 218 ± 17 μm in control rats. More details of the method are given by Olbrich et al. (1999).

After an equilibration period of 60 min to achieve a constant resting tone, vessels were preconstricted by infusion of 30 mM KCl (20 min) followed by treatment with KCl (30 mM) and 0.1 μM sodium nitroprusside (SNP; 20 min). After washout and reaching the preconstriction tone with 30 mM KCl alone, vessels were perfused with 30 mM KCl and 1 μM acetylcholine (ACh).

Statistics. All values are given as means ± S.E. of untreated age-matched control rats (n = 10), healthy control rats treated with trandolapril (n = 10), healthy control rats treated with LU 135252 (n = 9), untreated diabetic rats (n = 12), diabetic rats treated with trandolapril (n = 8), and diabetic rats treated with LU 135252 (n = 10). If necessary, the actual numbers (n) of the different variables are presented in the respective tables. Statistical analysis was performed using multivariant analysis of variance with disease as a two-step factor, treatment as a three-step factor, and the parameters measured as the dependent variables. If ANOVA indicated significant differences, Student's t test for paired or unpaired observations was performed at a level of significance of $P < .05$. For statistical analysis, we used the SYSTAT software (Jandel Scientific) and the SAS 6.12 Research Application 3.1 (SAS Institute, Heidelberg, Germany).

Chemicals. All chemicals used were of analytical grade and were purchased from Sigma (Deisenhofen, Germany). Trandolapril and LU 135252 were kindly provided by Knoll AG (Ludwigshafen, Germany).

Results

Blood Parameters (Plasma Glucose and Red Blood Cells) and Cataracts. In the normoglycemic groups of rats, no significant differences were found for the plasma glucose values before (week 0) and during chronic treatment (week 12 and week 23) with LU 135252 and trandolapril. In week 12, for example, the mean values ranged between 7.8 (141 mg/dl) and 8.5 mM (153 mg/dl). Three to four days after the administration of streptozotocin, all rats became considerably hyperglycemic with plasma glucose values more than 3 times higher than those in the control (week 0). In weeks 12 and 23, the three diabetic groups had plasma glucose values between 36 (649 mg/dl) and 46 mM (829 mg/dl) without significant differences among the groups. The untreated diabetic rats exhibited plasma glucose values approximately 5 times higher than those of control rats, indicating a pronounced hyperglycemia (for details see Table 1). An effect of the ET_A antagonist LU 135252 and the ACE inhibitor trandolapril on plasma glucose was not detectable in the normoglycemic or diabetic groups (Table 1).

Diabetic rats exhibited a marked anemia as indicated by reduced red blood cell count, which was significantly antagonized by both treatments. Red blood cell count was significantly reduced from 9.3 ± 0.19 × 10^6 erythrocytes/μl in control rats to 4.763 ± 0.28 × 10^6 erythrocytes/μl in diabetic rats ($P < .05$; values after 6 months). In control rats treated with either drug, red blood cell counts similar to those in untreated controls were seen (LU 135252, nondiabetic: 9.02 ± 0.48 × 10^6 erythrocytes/μl; trandolapril, nondiabetic: 9.19 ± 0.78 × 10^6 erythrocytes/μl). In diabetic rats treated with either drug, the reduction in red blood cell number was antagonized significantly (LU 135252, diabetic: 6.3 ± 0.35 × 10^6 erythrocytes/μl; trandolapril, diabetic: 6.33 ± 0.64 × 10^6 erythrocytes/μl, $P < .05$; Fig. 1). In addition, we found the characteristic cataracts in the eyes of the diabetic rats. No cataracts were found in nondiabetic animals, whereas 67% of the eyes of untreated diabetic rats exhibited a cataract. This was significantly reduced to 57% in trandolapril-treated rats ($P < .05$) and 50% in LU 135252-treated rats ($P < .05$).

Clinical Chemistry Variables. The plasma clinical chemistry parameters cholesterol, HDL, triglycerides, AST (glutamic-oxaloacetic transaminase), ALT (glutamic-pyruvic

TABLE 1

Plasma glucose (mM) of diabetic and nondiabetic rats before and during treatment with either trandolapril or LU 135252

Values are given as means ± S.E., n.

Group	Week 0	Week 12	Week 23
Control	9.3 ± 0.2[a] 10	7.8 ± 0.2[a] 9	7.7 ± 0.1[a] 10
LU 135252	9.4 ± 0.2[a] 10	8.5 ± 0.2[a] 10	8.4 ± 0.5[a] 9
Trandolapril	9.1 ± 0.2[a] 10	7.9 ± 0.2[a] 10	8.7 ± 0.2[a] 9
Diabetic	31.4 ± 0.9 12	39.0 ± 1.7[a] 12	39.1 ± 2.8 12
Diabetic + LU 135252	30.0 ± 0.6 11	45.5 ± 2.8 10	35.7 ± 1.5 9
Diabetic + trandolapril	29.3 ± 0.8 10	45.6 ± 2.5 6	42.3 ± 2.4 9

[a] Significant differences versus diabetic group ($P < .05$).

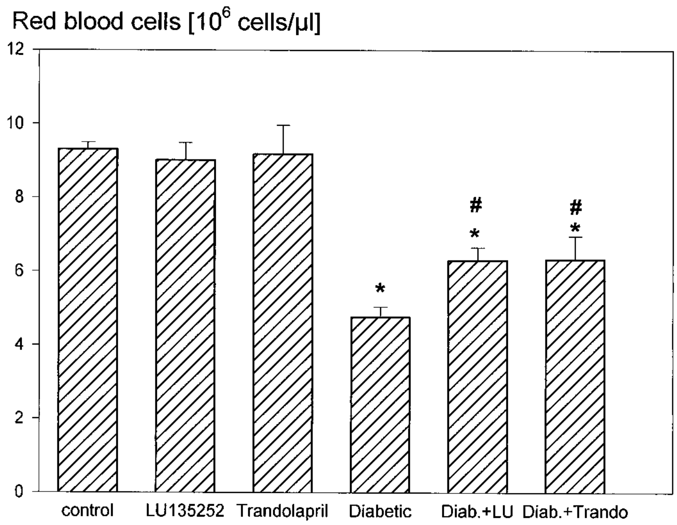

Fig. 1. Red blood cell counts (given as xx × 10^6 erythrocytes/μl) in nondiabetic and diabetic rats chronically (6 months) treated or untreated with either trandolapril or LU 135252. *, significant differences compared with the corresponding control group. #, significant changes against the diabetes mellitus group. For further details see *Results*. Diab., diabetic; LU, LU 135252; Trando, trandolapril.

transaminase), γ-GT, urea, and creatinine were determined in weeks 0 and 12 (values not shown) and in week 23 (Table 2). The untreated diabetic animals had slightly elevated cholesterol and HDL values (not significant) and unchanged triglycerides. After LU 135252 treatment of the diabetic rats, a tendency to lower cholesterol, HDL, and triglyceride values were observed. The ET_A antagonist treatment also led to an attenuation of the diabetic increase of liver enzymes that was significant for AST. In contrast, the ACE inhibitor trandolapril led to a further increase of AST, ALT, and γ-GT that was not significant due to large interindividual variation in this group. All diabetic groups had significantly elevated plasma urea values that were approximately 2 times higher than that of normoglycemic rats. The plasma creatinine values of the various experimental groups did not differ.

Body Weight. Although all normoglycemic animals showed a considerable increase in body weight within the experimental period of 23 weeks, the development of body weight was strongly retarded under diabetic conditions (Fig. 2). Body weight increase was only between 10 and 22% in the diabetic groups compared with 114 to 138% in normoglycemic animals. The increases in body weight were somewhat less pronounced (NS) in the two trandolapril groups.

Blood Pressure and Heart Rate. Systolic blood pressure and heart rate were monitored in weeks 8 and 15. In the normoglycemic animals, the ACE inhibitor trandolapril caused significant blood pressure lowering, whereas LU 135252 had no clear-cut effect on arterial pressure (Table 3). The untreated diabetic rats had slightly higher blood pressure values than the untreated normoglycemic controls. Both drug treatments caused a tendency to blood pressure lowering in the diabetic animals. The diabetes-induced blood pressure increase was associated with slightly lower heart rates, but the moderate blood pressure-lowering drug effects were not accompanied by increases in heart rate.

Kidney Function. Kidney function was analyzed in advanced diabetes mellitus (after 23 weeks). In the untreated diabetes group, a very pronounced urine production (polyuria) was observed that was about 20 times higher than that of the age-matched nondiabetic controls (Table 4). Trandolapril had no significant effect on water excretion in normoglycemic animals, but it slightly (although NS) reduced polyuria in diabetic animals. The ET_A antagonist, too, was without effect in nondiabetic rats, but it reduced the diabetic diuresis by approximately 50% ($P < .05$). All diabetic animals exhibited a pronounced proteinuria. In untreated diabetes, the protein loss was increased by a factor of approximately 6 compared with that in controls. Both drug treatments showed a tendency to reduce diabetic proteinuria. The creatinine clearance was only slightly affected after 23 weeks of diabetes, and a significant drug effect on this parameter was not observed.

Kidney Histology. With respect to renal histology, it became obvious that in diabetic rats the glomerular diameter was significantly increased, which was significantly antagonized by LU 135252 but not by trandolapril (Fig. 3a). Similar changes were found for the free width between the glomerular tufts and Bowman's capsule (Fig. 3b). In addition, a deposition of homogeneous eosinophilic material (hyaline)

TABLE 2

Plasma clinical chemistry variables in advanced diabetes mellitus with or without treatment with either trandolapril or LU 135252

Values are given as means ± S.E., *n*.

	Control	LU 135252	Trandolapril	Diabetic	Diabetic + LU 135252	Diabetic + Trandolapril
Cholesterol (mM)	2.1 ± 0.1 10	2.1 ± 0.2 9	2.1 ± 0.1 9	2.8 ± 0.5 12	2.1 ± 0.1 9	2.6 ± 0.1 9
HDL (mM)	1.5 ± 0.1 9	1.5 ± 0.2 8	1.3 ± 0.1 8	2.0 ± 0.3 10	1.5 ± 0.1 7	1.8 ± 0.1 6
Triglycerides (mM)	1.7 ± 0.1 10	1.0 ± 0.1[b] 9	1.1 ± 0.1[b] 9	1.6 ± 0.3 12	1.1 ± 0.2[b] 9	2.0 ± 0.3 9
AST (U/l)	52.2 ± 1.6[a] 10	53.2 ± 3.6[a] 9	53.3 ± 5.6[a] 9	97.3 ± 17.3 11	56.8 ± 8.6[a] 8	210.2 ± 78.8 8
ALT (U/l)	40.8 ± 3.5[a] 10	35.0 ± 1.7[a] 9	39.3 ± 3.4[a] 9	82.7 ± 9.6 12	54.9 ± 8.8 9	149.6 ± 46.4 8
γ-GT (U/l)	1.7 ± 0.3[a] 6	1.8 ± 0.4[a] 9	1.1 ± 0.6[a] 6	4.4 ± 0.9 10	3.0 ± 0.7 7	19.1 ± 9.1 7
Urea (mM)	6.8 ± 0.3[a] 9	7.6 ± 0.3[a] 9	7.7 ± 0.3[a] 9	15.9 ± 1.2 12	16.4 ± 0.9 9	17.7 ± 2.1 9
Creatinine (μM)	47.6 ± 1.1 10	49.9 ± 1.9 9	46.7 ± 0.9 10	45.3 ± 1.3 12	42.9 ± 3.0 9	45.9 ± 1.2 9

[a] Significant differences versus diabetic group.
[b] Significant changes versus nondiabetic controls.

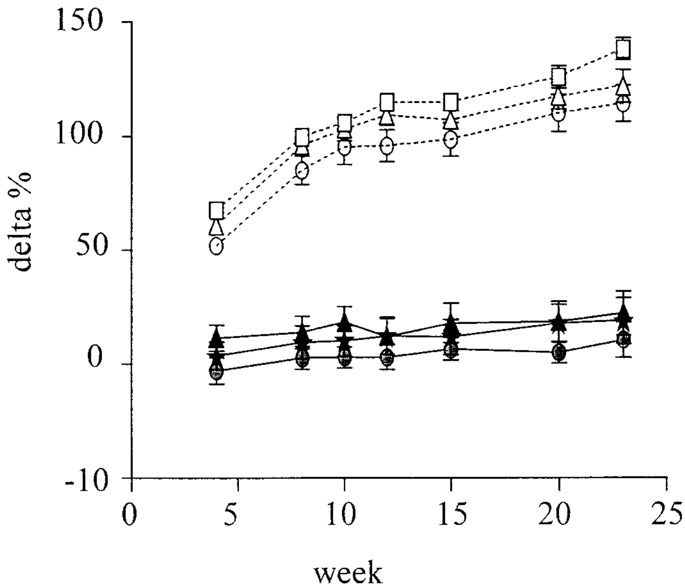

Fig. 2. Development of body weight in the course of the study. All diabetic animals exhibited significant lower body weight than the respective nondiabetic controls ($P < .05$). There was no significant influence of the treatment on this parameter. ★, diabetic; ▲, diabetic + LU 135252; ●, diabetic + trandolapril; □, control; △, LU 135252; ○, trandolapril.

TABLE 3

Systolic blood pressure (SAP; mm Hg) and heart rate (HR; min^{-1}) after 8 and 15 weeks in diabetic and nondiabetic rats and the influence of treatment with either trandolapril or LU 135252

Values are given as means ± S.E., *n*.

Group	Week 8		Week 15	
	SAP	HR	SAP	HR
Control	159 ± 4 10	376 ± 12 10	147 ± 3 10	348 ± 12 10
LU 135252	151 ± 5 10	355 ± 11 10	145 ± 4 10	343 ± 10 10
Trandolapril	133 ± 4[a] 10	368 ± 9 10	124 ± 6[a] 10	356 ± 9 10
Diabetic	171 ± 5 12	324 ± 7[a] 12	170 ± 6[a] 12	325 ± 9 11
Diabetic + LU 135252	152 ± 6 10	309 ± 12[a] 10	158 ± 12 8	323 ± 10 8
Diabetic + trandolapril	154 ± 7 10	336 ± 13[a] 10	149 ± 7 9	321 ± 13 8

[a] Significant differences versus controls ($P < .05$).

was seen within the glomeruli that was nearly completely prevented by LU 135252 but only slightly reduced by trandolapril (Fig. 3c).

Cardiac Histology. Regarding the cardiac histology, we found similar diameters of the cardiac muscle fibers in the range of 15 to 16 μm (control, 16 ± 0.26 μm; LU 135252 nondiabetic, 15.18 ± 0.1 μm; trandolapril nondiabetic, 15.49 ± 0.14 μm; diabetic, 15.63 ± 0.26 μm; LU 135252 diabetic, 15.3 ± 0.12 μm; trandolapril diabetic, 15.53 ± 0.21 μm). The differences among the groups were not significant. Thus, there was no cardiac hypertrophy in the diabetic animals.

Regarding the heart capillaries/muscle fibers ratio, we found 2.99 ± 0.56 capillaries/muscle fiber in nondiabetic control rats. This ratio was significantly decreased in diabetic animals and was fully prevented by both treatments (Fig. 4). In nondiabetic animals treated with either substance, an increase in ratio was also seen (Fig. 4).

TABLE 4

Kidney function control parameters in diabetic and nondiabetic rats and the influence of treatment with trandolapril or LU 135252

Values are given as means ± S.E., *n*.

Group	Urine	Proteinuria	Creatinine Clearance
	ml/24 h/kg	*mg/24 h/kg*	*100 g b.wt./min*
Control	15 ± 1[a] 10	28 ± 2[a] 9	0.43 ± 0.03 9
LU 135252	18 ± 3[a] 10	32 ± 3[a] 8	0.32 ± 0.04 7
Trandolapril	29 ± 5[a] 10	37 ± 4[a] 10	0.40 ± 0.02 10
Diabetic	360 ± 58 12	179 ± 35 8	0.38 ± 0.03 9
Diabetic + LU 135252	186 ± 47[a] 10	134 ± 23 10	0.39 ± 0.06 9
Diabetic + trandolapril	296 ± 50 9	127 ± 16 7	0.36 ± 0.05 7

[a] Significant differences versus diabetic group ($P < .05$).

Vascular Function of Mesenteric Artery. Regarding the functional response of mesenteric arteries, vessels of all experimental groups exhibited a marked vasoconstriction in response to KCl without differences among the groups (Fig. 5, top). Initial diameters did not differ between treated and untreated but, as in our previous studies (Olbrich et al., 1996, 1999), were enhanced in all diabetics (initial diameters: control, 218 ± 17 μm; LU 135252, 240 ± 29 μm; trandolapril, 223 ± 24 μm; diabetic, 446 ± 33* μm; diabetic + LU 135252, 409 ± 23* μm; diabetic + trandolapril, 459 ± 33* μm; *$P < .05$ versus nondiabetic control). Smooth muscular vasodilatation could be achieved with SNP to a similar degree in all experimental groups (Fig. 5, middle). However, dilatation after the administration of ACh was significantly impaired in diabetic animals (reduction of dilatation by approximately 50%). This impairment of ACh-induced vasodilatation was significantly antagonized by both chronic treatments (Fig. 5, bottom).

Discussion

In this study a typical streptozotozin-induced diabetes mellitus became evident as characterized by elevated blood glucose levels, cataracts, reduced body weight, anemia, proteinuria, renal hyaline deposition, glomerular widening, cardiac capillary rarefication, and endothelial dysfunction. Trandolapril and LU 135252 reduced (with some differences) diabetic alterations not only regarding nephropathy but also endothelial dysfunction, angiopathy, cataracts, and anemia. In the subsequent paragraphs, the results of our study relating to anemia and nephropathy will be discussed followed by a discussion of the results concerning angiopathy and endothelial dysfunction.

An interesting finding in diabetic animals was the anemia, which often is interpreted as a renal anemia (with reduced erythropoietin levels) due to the renal alterations or also has been suggested to be linked to autonomic neuropathy with erythropoietin depletion (Winkler et al., 1999). Both drugs improved red blood cell counts, which might be related to a generally improved vascular function, although the exact mechanism of this action cannot be elucidated in this study. The fact that trandolapril, although antagonizing this form of anemia, exhibited no or only minor effects on renal structure alterations might indicate that factors other than solely renal

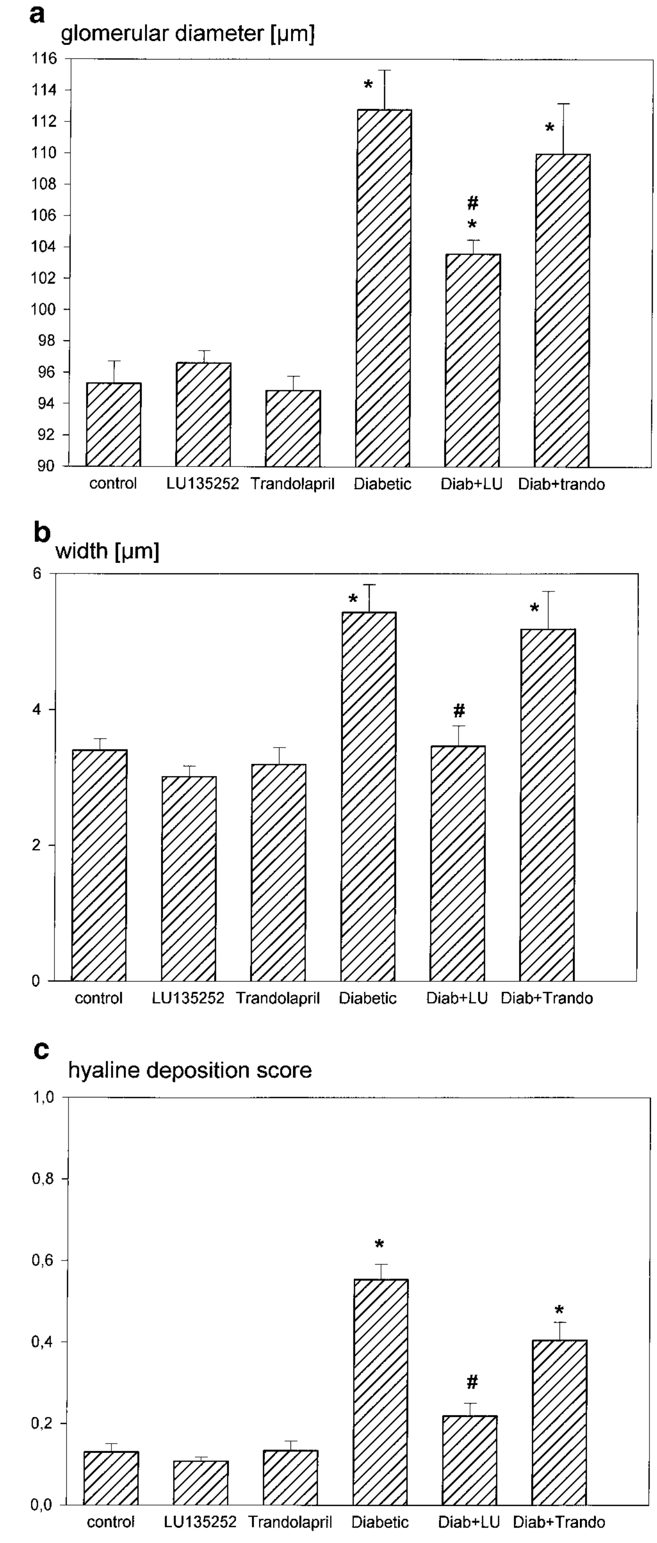

Fig. 3. a, glomerular diameter in kidneys of diabetic and nondiabetic rats that were chronically treated or untreated with either trandoplapril or LU 135252. b, free width between glomerular tufts and Bowman's capsule in renal glomeruli obtained from diabetic and nondiabetic rats that were chronically treated or untreated with either trandoplapril or LU 135252. c, deposition of homogeneous eosinophilic material, hyaline, in the renal glomeruli of diabetic and nondiabetic rats that were chronically treated or untreated with either trandoplapril or LU 135252. *, significant differences compared with the corresponding control group. #, significant changes against the diabetes mellitus group. For further details see *Results*. Diab, diabetic; LU, LU 135252; Trando, trandolapril.

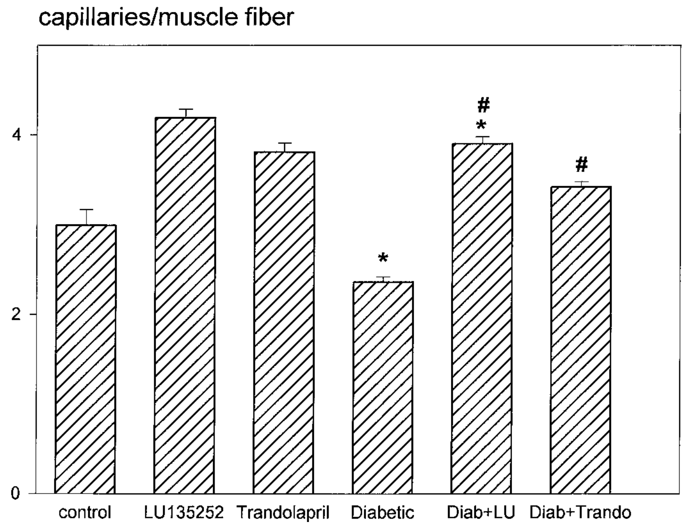

Fig. 4. Capillary/muscle fiber ratio in the left ventricles of diabetic and nondiabetic rats that were chronically treated or untreated with either trandoplapril or LU 135252. *, significant differences compared with the corresponding control group. #, significant changes against the diabetes mellitus group. For further details see *Results*. Diab, diabetic; LU, LU 135252; Trando, trandolapril.

structural alterations during diabetes mellitus may be involved in the genesis of this anemia. This is, to our knowledge, the first report of such an effect of ET_A blockade.

As far as the kidney is concerned, typical functional and histological changes were seen in this study. The pathogenesis of this diabetic nephropathy is still a matter of debate. However, it has been discussed that, in addition to advanced glycosylation end products (Shikata et al., 1995), osmotic diuresis and consecutive widening of the glomeruli might be involved in the mechanism underlying the activation of the renin-angiotensin system. The possible effectiveness of ACE inhibitors is demonstrated in the literature (Lewis et al., 1993; EUCLID Study Group, 1997; for review see Viberti and Chaturvedi, 1997) giving indirect evidence for the involvement of angiotensin in the pathophysiology of diabetic nephropathy. In our study, however, the effects of trandolapril on kidney function and histology were only small, with the exception of the significant effect on anemia. The smaller effect on kidney histology may indicate that the effects of trandolapril and LU 135252 involve different pathways. It has been shown that ACE inhibition reduces albuminuria by reducing the glomerular capillary pressure (Imanishi et al., 1997), which might be reflected by a slightly reduced polyuria and proteinuria in our study. However, an involvement of ET has also been supposed because, in patients, the elevation of plasma ET levels was associated with the onset of microalbuminuria (Neri et al., 1998). It has been shown using Northern blot analysis of ET-1 mRNA that the ET-1 gene is up-regulated in the diabetic kidney (Benigni et al., 1998). In addition to angiotensin II, ET has been described to be involved in extracellular matrix protein production (Ruiz-Ortega et al., 1994). Thus, the mRNA levels of certain extracellular matrix components, such as alpha 1(I), alpha 1(II), and alpha 1(III) collagen chains; laminin B1 and B2 chains; and certain growth factors, including tumor necrosis factor α, transforming growth factor β, and platelet-derived growth factor, are elevated in diabetic glomeruli. These levels can be reduced by ET_A antagonism with FR 139317 (Nakamura et al., 1995).

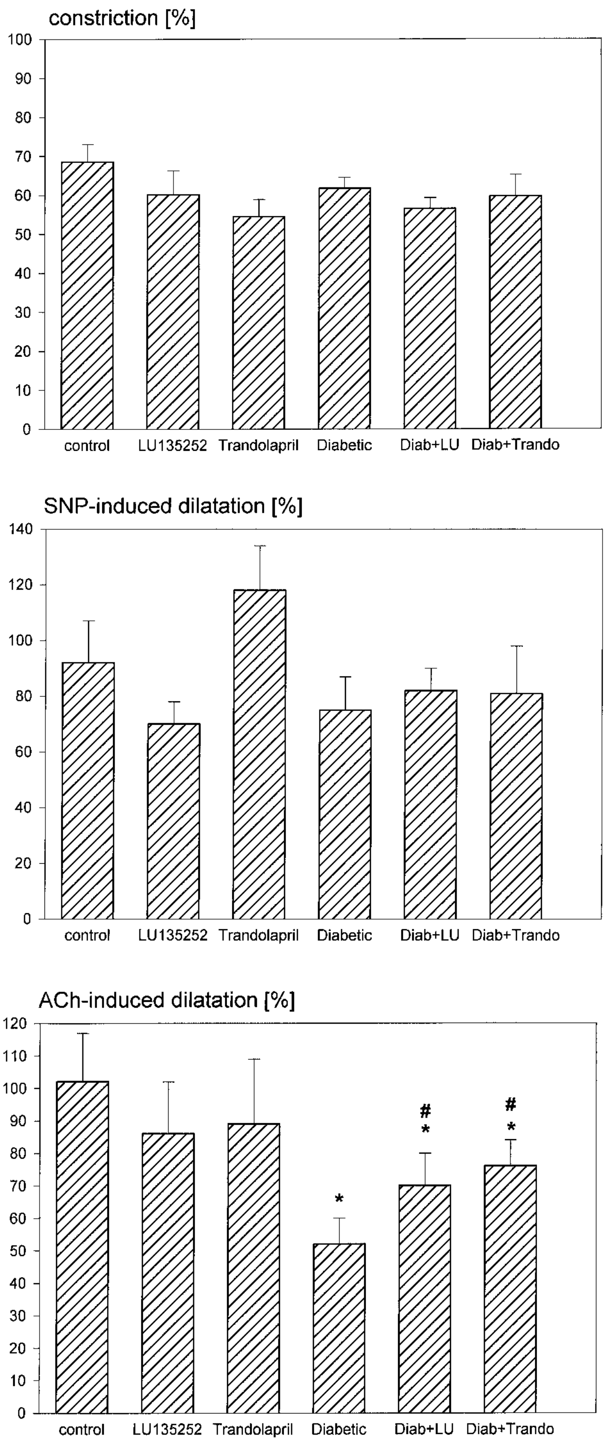

Fig. 5. Vascular response of G1 microvessels of the mesenteric vascular bed to KCl (30 mM) (top), SNP (0.1 μM) (middle), and ACh (1 μM) (bottom) as assessed by videomicroscopy of the isolated perfused (constant pressure) mesenterium of diabetic and nondiabetic rats that were chronically treated or untreated with either trandoplapril or LU 135252. The response to KCl is expressed as percentage of the initial control diameter; the response to SNP or ACh is expressed as percentage of dilatation of the foregoing constriction (i.e., 100% dilatation means that the constriction by KCl was completely antagonized). *, significant differences compared with the corresponding control group. #, significant changes against the diabetes mellitus group. For further details see *Results*. Initial diameters were: control, 218 ± 17 μm; LU 135252, 240 ± 29 μm; trandolapril, 223 ± 24 μm; diabetic, 446 ± 33 μm; diabetic + LU, 409 ± 23 μm; diabetic + trandolapril: 459 ± 33 μm. Diab, diabetic; LU, LU 135252; Trando, trandolapril.

Similarly, it became obvious in our study that the renal histological alterations (hyaline deposition, glomerular alterations) were prevented by the ET_A receptor antagonist LU 135252.

Regarding the alterations of kidney function, such as proteinuria, there was a reduction (in the order of 20–25%) by both drug treatments, although it did not reach full statistical significance ($P = .1$). It is, however, clinically known that functional changes do not correlate well with histological changes for which the reason is still unknown. It might be that positive effects can be seen in earlier disease states, although Benigni et al. (1998) observed a reduction of proteinuria under unselective ET receptor blockade even when the treatment started after the onset of proteinuria.

Another typical late complication of type I diabetes mellitus is angiopathy. A reduced NO release and endothelial dysfunction in diabetic rats has been demonstrated in earlier studies (Taylor and Poston, 1994; Taylor et al., 1995; Olbrich et al., 1996) and seems to be typical for this long-term model of type I diabetes mellitus. In this study, the endothelial dysfunction is reflected by a decreased dilatory response to ACh (which releases endogenous NO from the endothelium) in comparison with normal vasorelaxation in response to SNP, i.e., exogenously delivered NO, indicating a normal smooth muscular response to NO and unaltered constriction after KCl. Because the response to exogenous NO is not altered in diabetes but the response to ACh is, it can be concluded that endothelial release or production of NO is impaired in the diabetic animals. This is in good accordance to our previous investigation (Olbrich et al., 1996). The molecular basis of this reduction in NO liberation is still uncertain at present, although an altered signal transduction involving reduced Ca^{2+} signaling has been demonstrated in endothelial cell cultures chronically (5 days) exposed to enhanced glucose concentrations (Salameh and Dhein, 1998). As mechanisms for endothelial impairment, the production of free radicals (Tesfamariam, 1994), the activity of aldose reductase (Gonzalez et al., 1986), activation of protein kinase C (DeRubertis and Craven, 1994), and enhanced production of advanced glycosylation end products (Nakamura et al., 1993) as well as many other factors have been discussed. Recently, a reduction in ET_B receptor density has been shown to be involved in reduced NO liberation from diabetic rat kidney (Kakoki et al., 1999). This endothelial dysfunction was significantly improved by trandolapril as was previously seen with other ACE inhibitors (Cooper et al., 1994; Olbrich et al., 1996) and by the ET_A receptor antagonist LU 135252. To our knowledge, this is the first study demonstrating a positive effect of ET_A blockade on long-term diabetic endothelial dysfunction. An improvement of reduced neurovascular blood flow after ET_A blockade (but without investigation of endothelial function) has been seen in a short-term model (Cameron et al., 1994; Cameron and Cotter, 1996). The exact molecular mechanism by which ACE inhibitors or ET_A blockers interfere with diabetic endothelial dysfunction is still unclear.

A hypothesis could be that it is the blood pressure that contributes to the angiopathic changes and that it is the blood pressure-lowering drug effect itself that exerts protective effects. In this study, both drugs exhibited a minor, but not significant, effect on the elevated blood pressure in diabetic rats. Thus, elevated blood pressure might be a cofactor in the pathophysiology of this angiopathy. However, in a former study, it was shown that the antihypertensive agent metoprolol failed to antagonize diabetic angiopathic and nephropathic changes (Olbrich et al., 1999). An independence of the positive ET blockade effects from blood pressure was also shown with bosentan (Stevens and Tomlinson, 1995). In addition, the positive drug effects seem to be independent from blood glucose and blood cholesterol or body weight because these parameters were not affected by both treatments.

As a further indicator of a generalized diabetic angiopathy, we found a capillary rarefication in heart muscle in accordance to a previous study by our group (Olbrich et al., 1999). This was reversed by both treatments, indicating a possible role for angiotensin and ET in the pathophysiology of diabetic cardiomyopathy. Interestingly, both drugs exhibited a positive effect on capillary/muscle fiber ratio in nondiabetic hearts as well. At present, the molecular mechanisms underlying the regulation of capillary/muscle fiber ratio are not well understood. Future work has to be directed to that point.

In addition to antagonization of direct ET-1 effects, ET_A antagonists may interfere with the synergistic effects of angiotensin II and ET. Thus, it has been shown that the increase in vascular and renal ET-1 levels after angiotensin II administration could be prevented by LU 135252 (Barton et al., 1997).

In summary, the use of the ET_A selective blocker LU 135252 and the effects of this drug in our model, which can be characterized by a reduction in the incidence of cataracts, endothelial impairment, renal alterations, anemia, and cardiac capillary rarefication, indirectly indicate a pathophysiological role for ET that, at least in parts, seems to involve ET_A receptors.

Thus, our study demonstrates that ET_A receptor antagonization is effective against the typical type I diabetic late complications. Regarding renal histological changes, ET_A receptor antagonization was more effective than ACE inhibition. However, it should be mentioned that there are interspecies differences regarding the role of ET and ET_A receptors in regulation of renal function (Cernacek et al., 1998) so that one should be cautious not to transfer the findings of renal effects of ET_A blockade uncritically to other species. From the results of our study, we conclude that (1) both angiotensin and ET seem to contribute to the development of diabetic late complications and (2) in addition to ACE inhibition, blockade of ET_A receptors might be an interesting approach to antiangiopathic therapy.

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