ACE-inhibition is superior to endothelin A receptor blockade in preventing abnormal capillary supply and fibrosis of the heart in experimental diabetes

M.-L. Groß¹ · N. Heiß¹ · M. Weckbach¹ · A. Hansen² · A. El-Shakmak⁴ · A. Szabo⁴ · K. Münter⁵ · E. Ritz³ · K. Amann⁶

¹ Department of Pathology, University of Heidelberg, Heidelberg, Germany

² Department of Cardiology, University of Heidelberg, Heidelberg, Germany

³ Department of Internal Medicine, Nephrological Section, University of Heidelberg, Heidelberg, Germany

⁴ Department of Pediatric Nephrology, Semmelweis University Budapest, Budapest, Hungary

⁵ Bayer AG, Wuppertal, Germany

⁶ Department of Pathology, University of Erlangen, Erlangen, Germany

Abstract

Aims/hypothesis. There is little information whether cardiac capillary supply is deranged in diabetes. Hyperglycaemia is a potent stimulus for endothelin-1 (ET-1) production. We therefore hypothesised that increased ET-1 production in Streptozotocin-induced Type 1 diabetes causes abnormalities of cardiac capillaries and the aorta. To this end we compared the effects of an ET receptor A blocker (ET_A-RB) with that of an ACE-inhibitor (ACE-i) or their combination in rats with Streptozotocin (STZ) diabetes.

Methods. Sprague Dawley rats were injected with 65 mg STZ i.v. and subsequently developed diabetes. Rats were left untreated or received daily either the ACE-i Trandolapril, the ET_A-RB Darusentan or a combination of both. After 6 months the experiment was terminated and the heart and the aorta were investigated using quantitative morphological techniques.

Results. ACE-i but not ET_A-RB lowered blood pressure in STZ Type 1 diabetic rats. Capillary length density was lower in untreated STZ diabetic rats

(2932 ± 128 mm/mm³) compared to non-diabetic control rats (3410 ± 252 mm/mm³). Treatment with ACE-i (3568 ± 431 mm/mm³), but not with ET_A-RB (2893 ± 192 mm/mm³), prevented the decrease in capillary supply. Volume density of the myocardial interstitium was higher in untreated STZ diabetic rats ($0.86 \pm 0.04\%$) compared to non-diabetic control rats ($0.36 \pm 0.06\%$). In all three intervention groups the values were lower (ACE-i: $0.53 \pm 0.05\%$, ET_A-RB: $0.7 \pm 0.08\%$ and combination: 0.69 ± 0.1).

Conclusion/interpretation. Our study identifies a capillary defect of the heart in STZ diabetes, i.e. decreased capillary supply. This abnormality was reversed by ACE-i, but not by ET_A-R blockade. A similar trend, although not complete normalisation, was seen in cardiac fibrosis. [Diabetologia (2004) 47:316–324]

Keywords Diabetes mellitus · Animal model · Heart · Capillary · Fibrosis · Endothelin-1 · ACE-inhibitor · Endothelin A receptor blocker · Stereology · Hyperglycaemia

Received: 4 August 2003 / Revised: 22 October 2003

Published online: 15 January 2004

© Springer-Verlag 2004

M.-L. Groß (✉)

Department of Pathology, University of Heidelberg,
Im Neuenheimer Feld 220/221, 69120 Heidelberg, Germany
E-mail: Marie-Luise_Gross@med.uni-heidelberg.de

Abbreviations: ET-1, Endothelin1 · STZ, streptozotocin · ACE-i, ACE-inhibitor · ET_A-RB, ET receptor A blocker · AT1, Ang II receptor type 1 blockers · SD, Sprague Dawley

Diabetes mellitus is associated with high cardiac mortality. The heart of diabetic patients has a number of abnormalities which go beyond the widely held notion of coronary heart disease from accelerated atherogenesis [1, 2]. The spectrum of structural changes in so called “diabetic cardiomyopathy” [3] comprises, amongst others, left ventricular hypertrophy, cardiac interstitial fibrosis and metabolic abnormalities. Both the renin angiotensin system (RAS) and the endothelin system (ET) are thought to play a role in the pathogenesis of cardiac fibrosis and disturbed angiogenesis. In the heart ET-1 is produced by both cardiomyocytes

and endothelial cells, and cardiomyocytes express high affinity ET-1 binding sites [4, 5, 6]. ET-1 induces contraction and increases inotropy of cardiomyocytes. It also regulates salt and water homeostasis through actions on the kidney, stimulates the RAS and sympathetic nerve systems, contracts vascular smooth muscle cells and induces hypertrophy of vascular smooth muscle cells, mesangial cells and cardiomyocytes [7, 8]. ET-1 also mediates, at least in part, the chronic effects of Ang II on blood pressure [9, 10]. Conversely, ACE-inhibitors (ACE-i) and Ang II receptor type 1 blockers (AT1) diminish ET-1 expression [11, 12, 13]. In theory these findings would provide a strong rationale for the combination of endothelin receptor blockers (ET_A-RB) with ACE-i or AT1 receptor blockers.

ET-1 is responsible for the maintenance of basal vasomotor tone. Plasma ET-1 concentrations are increased in diabetic patients [14] and STZ-injected rats [15]. Whether increased ET-1 concentrations play a role in the genesis of structural changes of the heart has not yet been investigated. Evidence of a beneficial effect of ET on blood pressure and the heart has been provided in non-diabetic models [16, 17, 18, 19, 20].

Our aim was to compare the effect of a specific ET-A receptor antagonist with the well documented effects of an ACE-i (as well as the combination of both) on the structural lesion of the heart in an experimental model of Type 1 diabetes, with a particular focus on capillarisation, and volume of interstitial fibrous tissue.

Methods

Animals. Male Sprague Dawley rats (Charles River, Germany, mean body weight: 176 ± 19.8 g) were housed at constant room temperature (21°C) and humidity (75%) at a 12-h light and 12-h dark cycle. The rats had free access to water and standard rat pellets (Altromin 1324, Lage/Lippe, Germany). Experimental diabetes was induced by i.v. injection of 65 mg Streptozotocin (STZ) (Sigma Aldrich Chemie, Deisenhofen, Germany). Hyperglycaemia developed 2 days after STZ administration. Thereafter, diabetic rats were treated daily with 4.1 ± 1.4 IU/kg body weight (bw) of long acting human insulin (Insulin Ultratard HM, Aventis, Frankfurt, Main, Germany, 40 I.U./ml). We confirm, that we followed the 'Principles of Laboratory Animal Care'.

Experimental protocol. Four days after induction of diabetes the rats were randomly allocated to the following five experimental groups: (i) untreated non-diabetic control rats ($n=8$), (ii) untreated diabetic rats ($n=10$), (iii) diabetic rats + endothelin receptor blocker (ET_A-RB) (Darusentan, 50 mg/kg bw, $n=9$), (iv) diabetic rats + ACE-inhibitor (ACE-i) (Trandolapril, 0.3 mg/kg bw, $n=12$), (v) diabetic rats + ET_A-RB + ACE-i in combination (with doses as above, $n=12$).

Medications were administered in the drinking fluid at concentrations calculated to deliver the above mentioned doses. The concentrations of drugs in the drinking fluid were adjusted accordingly. Daily food and water consumption were monitored. Body weight, blood glucose and blood pressure (by tail plethysmography) were measured at regular intervals. After 3, 5 and 6 months the rats were kept in metabolic cages for a 24-h

urine collection. Urinary albumin excretion was measured using a rat specific sandwich ELISA system as described in detail elsewhere [21].

After 6 months the experiment was terminated by retrograde aortic perfusion with glutaraldehyde for morphometric and stereological investigations or with ice cold 0.9% NaCl for immunohistochemistry and in situ hybridisation, respectively [22]. Perfusion pressure was carefully monitored and adequacy of fixation was examined in pilot experiments.

Tissue preparation. After perfusion, the hearts of the rats were taken out to measure weight and volume. Tissue sampling and sectioning was done according to the orientator method: uniformly random sampling was achieved by preparing a set of equidistant slices of the left ventricle and the interventricular septum with a random start. Two slices were selected by area-weighted sampling and processed [22]. Eight pieces of the left ventricular wall, including the septum were embedded in Epon-Araldite. Semithin sections (1 µm) were cut, stained with methylene-blue and basic fuchsin and were examined by light microscopy with oil immersion and phase contrast at a magnification of 1000:1. After embedding, semithin sections were prepared and stained as described elsewhere [21].

Stereological analysis

Quantitative stereology: heart. The length density (L_v) of myocardial capillaries, i.e. the length of all capillaries per unit myocardial tissue volume, and the volume density (V_v) of cardiac capillaries, defined as the volume of capillaries per unit myocardial tissue volume, were measured in eight systematically subsampled areas per section ($57,600 \mu\text{m}^2$) using the point-counting method and a Zeiss eyepiece with 100 points for point counting (Integrationsplatte II; Zeiss, Oberkochen, Germany) [21]. The length density of myocardial capillaries (L_v) was determined by using the equation $L_v = 2 Q_A$. Q_A is the area density (i.e. the number of capillary profiles per area of myocardial reference tissue) [21].

Volume density (V_v) of interstitial tissue and myocytes was obtained using the point-counting method [21] according to the equation $P_p = V_v$. Inter-capillary distance was calculated according to a modification of the formula by [23].

Wall thickness and lumen diameter of intramyocardial arteries (diameter: 20–200 µm [24, 25]) were determined planimetrically using a semiautomatic image analysing system (Video-plan, Kontron, Eching, Germany). Wall-to-lumen ratio was calculated by dividing wall thickness and lumen diameter as described in detail [21].

Morphology of the aorta ascendens. Wall thickness of the aorta was measured on semithin sections using planimetry, as described in detail elsewhere [21].

Immunohistology

Paraffin sections were prepared and were reacted with the following antibodies using the avidin biotin method: Collagen IV (α-Collagen IV, polyclonal rabbit, Bio Trend, Cologne, Germany, 1:25) and ET-A receptor (sheep ETRA, Bio Trend Köln; 1:50) were investigated. We chose the ET-A receptor, because this receptor is predominantly found in the heart of rats [26]. ET-1 and TGF-β influence each other, therefore the expression of TGF-β (TGF-β1 polyclonal rabbit, Santa Cruz, Calif., USA) was analysed.

Table 1. Animal data

Animal group	Non-diabetic control (n=8)	Untreated diabetes (n=10)	Diabetes + ET _A -RB (n=9)	Diabetes + ACE-i (n=12)	Diabetes + combination (n=12)	ANOVA
Body weight (g)	521±52	301±52 ^a	322±46 ^a	323±29 ^a	350±73 ^a	<i>p</i> <0.05
Left ventricular weight/body weight ratio (mg/g)	2.15±0.18 ^b	4.06±0.24 ^a	3.72±0.22 ^{a,b}	3.41±0.29 ^{a,b}	3.78±0.42 ^a	<i>p</i> <0.05
Left ventricular/total heart weight ratio	0.71±0.06	0.68±0.06	0.7±0.05	0.67±0.07	0.69±0.05	NS
Blood glucose (mmol/l)	10.3±2.4	36.1±0.1 ^a	31.6±2.7 ^a	29.3±6.9 ^a	31.3±3.8 ^a	<i>p</i> <0.05
HbA _{1c} (%)	5±0.2	9.7±0.5 ^a	9.4±1.3 ^a	9.2±1.2 ^a	9.8±1.2 ^a	<i>p</i> <0.05
Daily food consumption (g)	37±3	58±6 ^a	50±8 ^a	47±3 ^a	51±9 ^a	<i>p</i> <0.05
Systolic bp (mmHg)	114±13	173±13 ^a	154±16 ^b	137±14 ^b	133±18 ^b	<i>p</i> <0.05
Albuminuria (mg/24 h)	0.48±0.15	1.73±0.56 ^a	1.06±0.38	0.68±0.11 ^b	0.56±0.15 ^b	<i>p</i> <0.05

Data are given as mean ± SD

^a *p*<0.05 vs non-diabetic control, ^b *p*<0.05 vs untreated diabetes

Cryostat sections that were 5 µm thick were prepared and were reacted with the following antibodies using the avidin biotin method [10]: Collagen I (α-Collagen I, polyclonal rabbit, Chemicon, Tenecula, Calif., USA, 1:2000) and ET-1 (α-ET-1 polyclonal rabbit, Biotrend, Cologne, Germany, 1:50). The concentration that was optimal for staining was evaluated testing different dilution series in a pilot study. Two investigators who were blinded with regard to the animal group did semiquantitative scoring as described in detail [22].

Semiquantitative RT-PCR for cardiac ET-1 and TGF-β mRNA expression

Total RNA (1 µg) was dissolved in 20 µl of a reaction mixture containing 4 mmol/l dATP, dCTP, dTTP, and dGTP; 10 U RNasin (Life Technologies BLR, Grand Island, N.Y., USA); 100 pmol random hexamers (Boehringer Mannheim, Mannheim, Germany); PCR buffer (final concentrations: 1.6 mmol/l (NH₄)₂SO₄, 2.0 mmol/l Tris-HCl [pH 8.4], 1.5 mmol/l MgCl₂); and 100 U Superscript reverse transcriptase (Life Technologies BLR). After incubation at 42°C for 45 min, the enzyme was denatured for 5 min at 94°C. Semiquantification of ET-1 and TGF-β mRNA was carried out using deletion mutants of the ET-1 and TGF-β cDNA as internal standard. The amplifications of the mutants resulted in PCR products of 300 bp for ET-1 and 302 bp for the TGF-β. The mutant cDNAs were added to the PCR mixture. Endogenous ET-1 0.4 pg or TGF-β 0.05 pg of plasmid respectively was added to the PCR mixture per sample. The PCR products were separated in a 3% metaphorge and scanned as TIF data. The quotient of measured endogenous sample and equivalent mutant was calculated by Scion-Image-program (National Institutes of Health, Bethesda, Md., USA).

In situ hybridisation for localisation of cardiac ET-1 and TGF-β mRNA expression

Because plasma ET-1 concentrations are increased in diabetes and antagonism of the ET system might be effective, mRNA expression of ET-1 was measured. ET-1 and TGF-β influence each other, subsequently mRNA expression of TGF-β was measured also. For in situ hybridisation, transversal cryostat

section of the myocardium (10 µm) were used. Probe preparation, labelling, hybridisation and development were carried out as described in detail elsewhere [22].

Statistics

All data are expressed as means ± SD. Kruskal-Wallis test and ANOVA, respectively, were chosen for analysis of variance, followed by the least-significant difference test to assess the differences between the groups. Results were considered significant if the *p* value was less than 0.05.

Results

Animal data. Untreated diabetic rats had stable, moderate hyperglycaemia throughout the 6 months of the study with a mean blood glucose concentration of 36.1±5.8 mmol/l and a mean HbA_{1c} of 9.7±0.5%. Blood glucose concentrations were not modified by either ACE-i or ET_A-RB treatment. Despite higher food intake (58±6 g/day) in diabetic compared to non-diabetic rats (37±3 g/day), body weight was lower in untreated diabetic rats. Body weight, as well as food or water intake were not modified by treatment with ACE-i or ET_A-RB or both combined (Table 1).

The relative weight of the left ventricle, i.e. left ventricular weight/body weight ratio, was higher in all diabetic groups compared to non-diabetic rats (*p*<0.05). It was lower (*p*<0.05), but not normalised, when diabetic rats were treated with the ET_A-RB and the ACE-i. Serum cholesterol and LDL cholesterol concentrations were not significantly (*p*<0.05) different between the experimental groups. Serum triglyceride concentrations were significantly higher in untreated diabetic rats (4.2±0.17 mmol/l) than in non-diabetic controls (1.06±0.35 mmol/l) and in treated diabetic rats (2.5±1.65 mmol/l ET_A-RB, 1.8±1.12 mmol/l ACE-i and 1.4±0.7 mmol/l combination).

After 6 months of diabetes, systolic blood pressure (bp) was higher in untreated diabetic rats compared to

Table 2. Stereological analysis of the heart

Animal group	Non-diabetic control (n=8)	Untreated diabetes (n=10)	Diabetes + ET _A -RB (n=9)	Diabetes + ACE-i (n=12)	Diabetes + combination (n=12)	ANOVA
L _v capillaries (mm/mm ³)	3410±252 ^b	2932±128 ^a	2893±181 ^{a,c}	3568±431 ^b	3477±329 ^b	<i>p</i> <0.05
Mean intercapillary distance (µm)	17.8±0.5 ^b	20.2±0.5 ^a	20.1±0.6 ^{a,c}	17.3±0.6 ^b	18.3±0.9 ^b	<i>p</i> <0.05
V _v interstitium (%)	0.36±0.06 ^b	0.86±0.04 ^a	0.70±0.08 ^{a,b,c}	0.53±0.05 ^{a,b}	0.69±0.10 ^{a,b}	<i>p</i> <0.05

Data are given as mean ± SD

^a *p*<0.05 vs non-diabetic control, ^b *p*<0.05 vs. untreated diabetes, ^c *p*<0.05 ET_A-RB treated diabetic rats vs. ACE-i treated rats

L_v: length density, V_v: volume density

non-diabetic control rats; it was lower in rats treated with ACE-i and with the combination. In diabetic rats treated with the ET_A-RB bp ranged between untreated and ACE-i treated diabetic rats, but was still significantly (*p*<0.05) lower than in untreated diabetic control rats.

Urinary albumin excretion as an index of renal dysfunction was higher in untreated diabetic than in non-diabetic rats (*p*<0.05). Urinary albumin excretion was reduced and nearly normalised by ACE-i and combination treatment, but not by ET_A-RB treatment.

Quantitative morphology of the myocardium (Fig. 1). Capillary length density was lower and mean intercapillary distance higher in untreated diabetic compared to non-diabetic control rats. Only ACE-i administered as mono therapy or in combination, but not ET_A-RB, prevented the abnormality of myocardial capillary supply in diabetes (Table 2, Fig. 1).

The volume density of the interstitial tissue was increased in untreated diabetic compared to control rats and treated diabetic rats indicating cardiac fibrosis. Again, the treatment with the ACE-i was associated with the most marked prevention of the increase in interstitial tissue.

Quantitative morphology of intramyocardial arterioles and of the aorta. The lumen diameter of intramyocardial arterioles was increased in untreated diabetic rats compared to non-diabetic control rats (*p*<0.05). Only ACE-i treatment alone or in combination prevented such arteriolar enlargement, but not ET_A-RB. Wall thickness of intramyocardial arterioles was increased in diabetic compared to control rats. It was lower in all intervention groups, but ACE-i in mono therapy or in combination, was more effective than ET_A-RB. This was also true for the changes in wall-lumen ratio which went in parallel (Table 3).

The wall thickness of the aorta was increased in untreated diabetic rats compared to non-diabetic control rats and all treated diabetic rats (*p*<0.05). The lumen diameter of the aorta was decreased in diabetic rats compared to control rats. Intervention with ACE-i mono therapy only significantly (*p*<0.05) improved data.

Immunohistochemical investigations. Cardiac capillary and myocyte ET-1 protein expression by semi-quantitative scoring was significantly (*p*<0.05) higher in untreated diabetic rats (0.8±0.2) compared to non-diabetic control rats (0.2±0.2). Only combination treatment reduced ET-1 protein expression (0.4±0.1; *p*<0.05), but not ET_A-RB (0.7±0.2) or ACE-i treatment (0.6±0.3).

ET-A receptor protein expression by semiquantitative scoring was higher (*p*<0.05) in capillary endothelial cells and myocytes of untreated diabetic rats (2.0±0.3) compared to non-diabetic control rats (0.5±0.3). In diabetic rats ET-A receptor expression was lower (*p*<0.05) after ACE-i (1.4±0.3) or combination treatment (1.3±0.3), but not ET_A-RB treatment (1.8±0.4).

TGF-β protein expression was significantly higher (*p*<0.05) in untreated diabetic (1.3±0.4) and ET_A-RB (1.3±0.3) treated rats compared to decreased expression in non-diabetic control (0.5±0.3), ACE-i treated (0.5±0.3) and combination treated diabetic rats (0.6±0.1).

Collagen IV protein expression was higher (*p*<0.05) in untreated diabetic (1.01±0.5) compared to non-diabetic rats (0.07±0.01). All interventions reduced expression for collagen IV, but ACE-i and combination treatment were more effective (diabetes + ET_A-RB: 0.43±0.06; diabetes + ACE-i: 0.25±0.14; diabetes + combination: 0.19±0.01).

Collagen I protein expression was not significantly (*p*<0.05) different between experimental groups.

Semiquantitative RT-PCR for cardiac ET-1 and TGF-β mRNA expression. Relative ET-1 mRNA expression was increased (*p*<0.05) in the heart of untreated diabetes compared to non-diabetic control rats. Cardiac ET-1 mRNA expression was lower (*p*<0.05) in diabetic rats on combination treatment, but not in ACE-i and ET_A-RB treated diabetic rats (Fig. 2).

In parallel, cardiac TGF-β mRNA was higher (*p*<0.05) in untreated diabetic rats compared to non-diabetic control rats. All interventions led to statistically significant lower TGF-β mRNA levels, but they were normalised only in the combination group.

Fig. 1a–d. Normal capillary supply in a non-diabetic SD rat (a). Reduced density of capillary profiles and expansion of the nonvascular interstitium is clearly visible in the myocardium of an untreated diabetic rat (b). Myocardium of diabetic rats

treated with ET_A-RB (c) or combination treatment (d) with almost normal myocardial capillary density. Semithin sections, mag.: 1:1200

Table 3. Measurements of intramyocardial arterioles and of the aorta thoracica descendens

Animal group	Non-diabetic control (n=8)	Untreated diabetes (n=10)	Diabetes + ET _A -RB (n=9)	Diabetes + ACE-i (n=12)	Diabetes + combination (n=12)	ANOVA
Wall thickness of intramyocardial arteries (μm)	3.24±0.46 ^b	4.88±0.59 ^a	4.41±0.52 ^{a,b,c}	3.63±0.55 ^{a,b}	3.63±0.59 ^{a,b}	<i>p</i> <0.05
Lumen diameter of Intramyocardial arteries (μm)	34.0±3.37 ^b	39.9±5.30 ^a	38.1±5.25 ^a	37.1±5.15 ^{a,b}	37.7±4.3 ^b	<i>p</i> <0.05
Wall-to-lumen ratio of intramyocardial arteries (×10 ⁻¹)	0.95±0.08 ^b	1.14±0.12 ^a	1.03±0.12 ^{a,b,c}	0.94±0.12 ^b	0.92±0.14 ^b	<i>p</i> <0.05
Wall thickness of the aorta (μm)	93.8±7.25 ^b	104±6.82 ^a	93.8±6.32 ^b	87.2±5.09 ^b	92.5±12.06 ^b	<i>p</i> <0.05
Lumen diameter of the aorta (μm)	1319±199 ^b	1045±114 ^a	1031±40 ^{a,c}	1304±104 ^b	983±176 ^{a,b}	<i>p</i> <0.05
Wall-to-lumen ratio aorta (×10 ⁻³)	7.63±1.15 ^b	10.9±2.06 ^a	7.53±0.85 ^b	6.90±0.78 ^b	9.4±3.37 ^a	<i>p</i> <0.05

Data are given as mean ± SD

^a *p*<0.05 vs non-diabetic control, ^b *p*<0.05 vs untreated diabetes, ^c *p*<0.05 ET_A-RB treated diabetic rats vs ACE-i treated

In situ hybridisation (Fig. 3). ET-1 mRNA expression of interstitial and capillary endothelial cells (Fig. 3) was increased ($p<0.05$) in the heart of untreated diabetic rats compared to non-diabetic control rats. Neither treat-

ment with ACE-i nor with ET_A-RB or the combination lowered ET-1 gene expression. TGF- β mRNA expression of interstitial cells was increased ($p<0.05$) in diabetic rats. All interventions lowered the expression.

Fig. 2. Effect of ET_A-RB and ACE-i on expression of ET-1 and TGF- β mRNA by semiquantitative RT-PCR

Discussion

Our study identifies decreased capillary supply as well as increased mean intercapillary distance as a new finding and confirmed marked interstitial fibrosis in the heart in experimental Type 1 STZ-induced diabetes. As to the pathomechanisms involved, it is of interest that the expression of collagen IV, ET-1 and TGF- β was increased in untreated STZ diabetic rats. Treatment with an ACE-i, but not with ET_A-RB, abrogated both the capillary abnormality and the interstitial fibrosis. Combination of ACE-i and ET_A-RB had no additive beneficial effect.

Fig. 3a–d. Expression of ET mRNA (brown signal) by non-radioactive in situ hybridisation (magnification 1:400). Only moderate expression in the heart of an untreated non-diabetic control rat (a), in comparison to a marked increase in an un-

treated diabetic rat (b). Intervention with ET_A-RB monotherapy (c) and combination treatment with ET_A-RB and ACE-i (d) lowered ET mRNA expression in diabetes

The finding of diminished capillary-length density reflects the failure of the capillary growth and the failure to increase *pari passu* with the increase of cardiomyocyte volume, creating capillary/cardiomyocyte mismatch. The increased mean intercapillary distance implies that the distance from the centre of the capillaries to the centre of the cardiomyocyte, and by implication for oxygen diffusion, is increased. Although the study does not provide direct evidence, it seems logical that diminished capillary supply is at least one major cause contributing to the impaired ischaemia tolerance of the heart in diabetes.

We also confirmed previous findings of interstitial fibrosis i.e. non-replacement type of fibrosis. In the replacement type of fibrosis, fibrous tissue substitutes for vanished necrotic parenchyma. In contrast, the type of fibrosis we documented in the STZ diabetic heart constitutes primary fibrosis resulting from activation of interstitial fibroblasts, similar to what has been documented in rats exposed to aldosterone [27] or Ang II [28]. Cardiac fibrosis impairs left ventricular compliance, causes an abnormal stress relationship and (particularly in conjunction with diminished blood supply) interferes with the electrical stability of the heart [29].

As to the pathomechanisms involved, we found increased expression of ET-1, ET receptor, TGF- β and collagen IV in untreated STZ diabetic rats, confirming, at least in part, data of other laboratories [30, 31, 32]. A further finding of potential pathogenetic relevance was increased systolic blood pressure measured by tail plethysmography.

The influence of ET_A-RB on bp is controversial, but several confounders make the comparison of the reported data difficult e.g. different administered doses, differences in animal models used and differences in bp measurement techniques [33, 34, 35]. Of note, ET_A-RB lowered bp in diabetic rats in our study. The maximal decrease in bp was seen with combination treatment. The increase in systolic bp in diabetes mellitus could be related to the stimulatory effect of hyperglycaemia on the RAS [36, 37, 38] or generation of oxygen radical species reducing the availability of nitric oxide [39]. A normalisation of systemic bp in non-selective ET-1 blockade in diabetic rats is described [40]. At the same dose of Darusentan, no bp lowering was noted in rats with surgical ablation of the kidney [19, 41] further suggesting that the role of ET is specific for diabetes. A small but sustained reduction of 24-h blood pressure with Darusentan was noted, whereas endothelin-dependent vasorelaxation remained unchanged [42].

Using a two dimensional approach a significant increase of cardiac capillaries in STZ rats after ET_A-RB treatment is noted [33]. This finding is in contrast to our results using a stereological technique permitting analysis in three dimensional space. The above results using conventional methodology could easily have

been flawed, however, by the anisotropy of cardiac tissue.

The ET receptor antagonist abrogated dysfunction of cultured endothelial cells exposed to high glucose concentrations [43]. It also has been shown that ET-1 mRNA receptor binding is increased in the hearts of diabetic rats [5, 44], similar to what is found in cardiac disease [13]. In the present long-term study increased ET-1 and ETA receptor expression could also be demonstrated in diabetic rats. ET-1 expression was decreased only by combination therapy. In contrast, increased expression of ET-A receptor was also reduced by ACE-i (and combination) therapy, but not by ET_A-RB. This observation suggests a major role of the RAS in the up-regulation of the ET system in the heart of diabetic rats. Data reported in the literature are consistent with a high vascular ACE activity, which could be an important factor for the progressive deterioration of vascular function in STZ rats [45].

It is not clear why the ET_A-RB was less effective to prevent capillary deficit in the heart, although the ET-1 system is up-regulated in diabetes. One explanation proposed by some authors is that damaged cardiomyocytes are unable to synthesise ET-1 in response to Ang II stimulation [13, 39, 46]. For diabetes this explanation is excluded by our results. We cannot exclude, however, post receptor abnormalities in diabetes, so that ETA receptor stimulation fails to yield an appropriate response. It has been speculated that an activated local RAS is responsible for an increased AT₂ receptor mediated response to ANG II in cardiomyocytes and subsequent apoptosis [46]. This hypothesis could explain the superior efficacy of the ACE-i in our study. Interestingly, in diabetes apoptosis of cardiomyocytes seem to occur independent of hypertension [39].

We found that ET_A-RB lowered cardiac interstitial fibrosis and wall thickness of the aorta. The volume density of the interstitium was lower in all intervention groups. ACE-i, but not ET_A-RB prevented the increase in wall thickness of intramyocardial arteries. However, the wall-to-lumen ratio of intramyocardial arteries was also reduced by ET_A-RB compared to non-diabetic rats. The increase in aortic wall thickness was prevented by ET_A-RB and ACE-i suggesting a role for endogenous ET in the pathogenesis of vessel disease in diabetes. Additionally, ET_A-RB prevented bp increase and vascular remodelling in large and small vessels in a model of salt-sensitive hypertension [47].

The relationship between ET-1 and TGF- β is complex: ET-1 stimulates TGF- β synthesis [48], and in turn TGF- β augments ET-1 expression as part of a positive feedback cycle [49]. In contrast to results of others [50], in our study ET_A-RB reduced TGF- β mRNA expression as potently as the ACE-i. The combination of the two interventions provided no further benefit. Diabetes was associated with increased TGF-

β mRNA expression. Such overexpression was reduced by ET_A-RB and by ACE-i. This finding confirms that ET-1 increases TGF- β , at least in this model, and this could be one mechanism causing the increase in interstitial tissue and arterial wall thickness. On the other hand ET-1 mRNA was increased in diabetic rats and this increase was only prevented by combination treatment.

The modification of extracellular matrix components by advanced glycation leads to increased collagen cross linking and increased myocardial stiffness in diabetes [31]. In addition our study showed increased expression for collagen IV in diabetic rats. All interventions reduced its expression, but ACE-i and combination treatment were more effective than the ET_A-RB.

In conclusion, this study identifies a myocardial capillary deficit in diabetes. Furthermore, treatment with ACE-i was more effective than blockade of the ET-system in preventing interstitial fibrosis and capillary defect of the heart in diabetic rats. This observation documents a major pathophysiological role of the RAS in the pathogenesis of the cardiac abnormality, but the ET system seems to play a certain role in the genesis of diabetic vessel disease.

Acknowledgements. M.L. Groß was recipient of a grant in the Graduiertenkolleg "Nieren- und Kreislaufregulation" of the Deutsche Forschungsgemeinschaft (DFG). The study was supported by grants of the Deutsche Diabetesstiftung and the University of Heidelberg (Juniorprojects No. 83/97 and 133/00). The skillful technical assistance of Z. Antoni, P. Rieger and H. Ziebart is gratefully acknowledged. Special thanks to H. Derks and U. Burkhardt for organising the photographs and the layout.

References

1. Fein FS, Sonnenblick EH (1994) Diabetic cardiomyopathy. *Cardiovasc Drugs Ther* 8:65–73
2. Fischer M, Wiest G, Tekesin I et al. (1992) Effects of combined renovascular hypertension and diabetes mellitus on myocardial cells, non-vascular interstitium and capillaries: a stereological study on rat hearts. *Virchows Arch A Pathol Anat Histopathol* 420:499–506
3. Standl E, Schnell O, Balletshofer B et al. (1997) Influence of diabetes mellitus on the heart and macrovascular mortality. *Diabetologia* 40 [Suppl 2]:S125–S126
4. Suzuki T, Kumazaki T, Mitsui Y (1993) Endothelin-1 is produced and secreted by neonatal rat cardiac myocytes in vitro. *Biochem Biophys Res Commun* 191:823–830
5. Chua BH, Chua CC, Diglio CA, Siu BB (1998) Regulation of endothelin-1 mRNA by angiotensin II in rat heart endothelial cells. *Biochim Biophys Acta* 1178:201–206
6. Fukuchi M, Giaid A (1998) Expression of endothelin-1 and endothelin-converting enzyme-1 mRNAs and proteins in failing human hearts. *J Cardiovasc Pharmacol* 31 [Suppl 1]:S421–S423
7. Haynes WG, Ferro CJ, Webb DJ (1998) Bosentan in essential hypertension. *N Engl J Med* 339:346–347
8. Haynes WG, Webb DJ (1998) Endothelin as a regulator of cardiovascular function in health and disease. *J Hypertens* 16:1081–1098
9. Rabelink TJ, Strokes ES, Bouter KP, Morrison P (1998) Endothelin blockers and renal protection: a new strategy to prevent end-organ damage in cardiovascular disease? *Cardiovasc Res* 39:543–549
10. Rabelink TJ, Bakris GL (1998) The renin-angiotensin system in diabetic nephropathy: the endothelial connection. *Miner Electrolyte Metab* 24:381–388
11. Disashi T, Nonoguchi H, Iwaoka T et al. (1997) Endothelin converting enzyme-1 gene expression in the kidney of spontaneously hypertensive rats. *Hypertension* 30:1591–1597
12. Verhaar MC, Strachan FE, Newby DE et al. (1998) Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation* 97:752–756
13. Sernerri GG, Cecioni I, Vanni S et al. (2000) Selective upregulation of cardiac endothelin system in patients with ischemic but not idiopathic dilated cardiomyopathy: endothelin-1 system in the human failing heart. *Circ Res* 86:377–385
14. Takahashi K, Ghatei MA, Lam HC, O'Halloran DJ, Bloom SR (1999) Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia* 33:306–310
15. Misurski DA, Hopfner RL, McNeill JR, Gopalakrishnan V (1999) Endothelium-dependent alterations in responses of the mesenteric vascular bed in the streptozotocin diabetic rat. *Proc West Pharmacol Soc* 42:49–51
16. Fica S, Herizi A, Mimran A, Jover B (2001) Endothelin blockade in angiotensin II hypertension: prevention and treatment studies in the rat. *Clin Exp Pharmacol Physiol* 28:1100–1103
17. Gurbanov K, Rubinstein I, Hoffman A, Abassi Z, Better OS, Winaver J (1996) Bosentan improves renal regional blood flow in rats with experimental congestive heart failure. *Eur J Pharmacol* 310:193–196
18. Marin-Garcia J, Goldenthal MJ, Moe GW (2002) Selective endothelin receptor blockade reverses mitochondrial dysfunction in canine heart failure. *J Card Fail* 8:326–332
19. Nabokov AV, Amann K, Wessels S, Munter K, Wagner J, Ritz E (1999) Endothelin receptor antagonists influence cardiovascular morphology in uremic rats. *Kidney Int* 55:512–519
20. Oie E, Clausen OP, Yndestad A, Groggaard HK, Attramadal H, Oie E (2002) Endothelin receptor antagonism attenuates cardiomyocyte apoptosis after induction of ischemia in rats. *Scand Cardiovasc J* 36:108–116
21. Amann K, Wolf B, Nichols C (1997) Aortic changes in experimental renal failure: hyperplasia or hypertrophy of smooth muscle cells? *Hypertension* 29:770–775
22. Amann K, Munter K, Wessels S (2000) Endothelin A receptor blockade prevents capillary/myocyte mismatch in the heart of uremic animals. *J Am Soc Nephrol* 11:1702–1711
23. Henquell L, Odoroff CL, Honig CR (1997) Intercapillary distance and capillary reserve in hypertrophied rat hearts beating in situ. *Circ Res* 41:400–408
24. Buzello M, Boehm C, Orth S (2003) Myocyte loss in early left ventricular hypertrophy of experimental renovascular hypertension. *Virchows Arch* 442:364–371
25. Rodriguez-Porcel M, Lerman A, Ritman EL, Wilson SH, Best PJ, Lerman LO (2000) Altered myocardial microvascular 3D architecture in experimental hypercholesterolemia. *Circulation* 102:2028–2030
26. Kirchengast M, Munter K (1999) Endothelin-1 and endothelin receptor antagonists in cardiovascular remodeling. *Proc Soc Exp Biol Med* 221:312–325
27. Weber KT, Anversa P, Armstrong PW et al. (1992) Remodeling and reparation of the cardiovascular system. *J Am Coll Cardiol* 20:3–16

28. Weber KT, Brilla CG, Campbell SE, Guarda E, Zhou G, Sriram K (1993) Myocardial fibrosis: role of angiotensin II and aldosterone. *Basic Res Cardiol* 88 [Suppl 1]:107–124
29. Weber KT, Brilla CG (1993) Structural basis for pathologic left ventricular hypertrophy. *Clin Cardiol* 16:II10–II14
30. Riva E, Andreoni G, Bianchi R (1998) Changes in diastolic function and collagen content in normotensive and hypertensive rats with long-term streptozotocin-induced diabetes. *Pharmacol Res* 37:233–240
31. Candido R, Forbes JM, Thomas MC et al. (2003) A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes. *Circ Res* 92:785–792
32. Ayaz M, Can B, Ozdemir S, Turan B (2002) Protective effect of selenium treatment on diabetes-induced myocardial structural alterations. *Biol Trace Elem Res* 89:215–226
33. Dhein S, Hochreuther S, Aus Dem Spring C, Bollig K, Hufnagel C, Raschack M (2000) 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. *J Pharmacol Exp Ther* 293:351–359
34. Cavašin MA, Carretero OA, Yang F, Oja-Tebbe N, Peng H, Yang XP (2002) Long-term effects of selective and nonselective endothelin receptor antagonists in mice with heart failure. *J Card Fail* 8:254–261
35. Basso C, Thiene G, Della Barbera M, Angelini A, Kirchengast M, Illiceto S (2002) Endothelin A-receptor antagonist administration immediately after experimental myocardial infarction with reperfusion does not affect scar healing in dogs. *Cardiovasc Res* 55:113–121
36. Mathiesen ER, Borch-Johnsen K, Jensen DV, Deckert T (1989) Improved survival in patients with diabetic nephropathy. *Diabetologia* 32:884–886
37. Hong SW, Isono M, Chen S, Iglesias-De La Cruz MC, Han DC, Ziyadeh FN (2001) Increased glomerular and tubular expression of transforming growth factor-beta1, its type II receptor, and activation of the Smad signaling pathway in the db/db mouse. *Am J Pathol* 158:1653–1663
38. Woods LL, Mizelle HL, Hall JE (1987) Control of renal hemodynamics in hyperglycemia: possible role of tubuloglomerular feedback. *Am J Physiol* 252:F65–F73
39. Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ (2002) Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. *Diabetes* 51:1938–1948
40. Benigni A, Colosio V, Brena C, Bruzzi I, Bertani T, Remuzzi G (1998) Unselective inhibition of endothelin receptors reduces renal dysfunction in experimental diabetes. *Diabetes* 47:450–456
41. Nabokov A, Amann K, Wagner J, Gehlen F, Munter K, Ritz E (1996) Influence of specific and non-specific endothelin receptor antagonists on renal morphology in rats with surgical renal ablation. *Nephrol Dial Transplant* 11:514–520
42. Witte K, Reitenbach I, Stolpe K, Schilling L, Kirchengast M, Lemmer B (2003) Effects of the endothelin A receptor antagonist darusentan on blood pressure and vascular contractility in type 2 diabetic Goto-Kakizaki rats. *J Cardiovasc Pharmacol* 41:890–896
43. Verma S, Maitland A, Weisel RD et al. (2002) Hyperglycemia exaggerates ischemia-reperfusion-induced cardiomyocyte injury: reversal with endothelin antagonism. *J Thorac Cardiovasc Surg* 123:1120–1124
44. Vesci L, Mattera GG, Tobia P, Corsico N, Calvani M (1995) Cardiac and renal endothelin-1 binding sites in streptozotocin-induced diabetic rats. *Pharmacol Res* 32:363–367
45. Crespo MJ, Moreta S, Gonzalez J (2003) Cardiovascular deterioration in STZ-diabetic rats: possible role of vascular RAS. *Pharmacology* 68:1–8
46. Fiordaliso F, Li B, Latini R (2000) Myocyte death in streptozotocin-induced diabetes in rats in angiotensin II-dependent. *Lab Invest* 80:513–527
47. Park JB, Schiffrin EL (2001) ET(A) receptor antagonist prevents blood pressure elevation and vascular remodeling in aldosterone-infused rats. *Hypertension* 37:1444–1449
48. Zoja C, Orisio S, Perico N (1991) Constitutive expression of endothelin gene in cultured human mesangial cells and its modulation by transforming growth factor-beta, thrombin, and a thromboxane A2 analogue. *Lab Invest* 64:16–20
49. Kurihara H, Yoshizumi M, Sugiyama T et al. (1998) Transforming growth factor-beta stimulates the expression of endothelin mRNA by vascular endothelial cells. *Biochem Biophys Res Commun* 159:1435–1440
50. Gilbert RE, Rumble JR, Cao Z (2000) Endothelin receptor antagonism ameliorates mast cell infiltration, vascular hypertrophy, and epidermal growth factor expression in experimental diabetes. *Circ Res* 86:158–165