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Cardioprotective effects of atrasentan, an endothelin-A receptor antagonist, but not of nitric oxide in diabetic mice with myocyte-specific overexpression of endothelial nitric oxide synthase

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1 We investigated the roles of nitric oxide (NO) and endothelin-1 (ET-1) in organ dysfunction in diabetic mice with normal genotype (wild-type, WT) or myocyte-specific overexpression of endothelial NO synthase (eNOS) (transgenic, TG) after chronic oral treatment with the endothelin-A (ET_A) receptor antagonist atrasentan.

2 Mice were rendered diabetic by injection of 200 mg kg^{-1} streptozotocin (STZ). Experimental groups were: untreated WT diabetic ($n=9$), untreated TG diabetic ($n=9$), atrasentan-treated WT diabetic ($n=9$), atrasentan-treated TG diabetic ($n=8$) and the four corresponding nondiabetic groups ($n=5$). Atrasentan was administered orally *via* drinking water at 3 mg kg^{-1} per day over 28 days. All diabetic mice developed similar hyperglycaemia (27–30 mmol l^{-1}).

3 Atrasentan treatment significantly improved left ventricular systolic and diastolic function in response to exogenous norepinephrine, but there were no differences between genotypes.

4 Atrasentan antagonized the diabetic impairments in endothelium-dependent coronary relaxation and thromboxane-receptor mediated aortic constriction. Further, it improved cardiac and renal oxidant status as evident from reduced tissue malondialdehyde levels.

5 Atrasentan reduced diabetic urine flow, proteinuria and plasma creatinine levels, but creatinine clearance was not significantly altered.

6 These results suggest that in experimental type 1 diabetes, blocking ET_A receptors ameliorates myocardial, coronary and renal function and improves tissue oxidant status, whereas raising myocardial NO levels has neither beneficial nor deleterious effects on diabetic cardiomyopathy in this transgenic model.
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Abbreviations: $+dP/dt$ and $-dP/dt$, maximal rates of left ventricular pressure rise and decline; eNOS, endothelial NO synthase; ET, endothelin; iNOS, inducible NOS; LVDevP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; NO, nitric oxide; STZ, streptozotocin; TG, transgenic; WT, wild-type

Introduction

The establishment of hyperglycaemia as a key initiating factor for the development of chronic diabetic complications has been a milestone in diabetes research. Vascular endothelial cells, cardiac myocytes and the kidneys are important targets of hyperglycaemic damage in patients with type 1 and type 2 diabetes (Sheetz & King, 2002). Hyperglycaemia is known to impair endothelium-dependent regulation of vascular tone, to promote the development of diabetic cardiomyopathy with impaired systolic and diastolic function, and to exacerbate end-stage renal disease (Mäkimattila *et al*., 1996; Ritz & Orth, 2000; Tang & Young, 2001). The biochemical abnormalities occurring secondary to hyperglycaemia include oxidative stress reactions and activation of protein kinase C (Koya & King, 1998). These changes may contribute significantly to increased morbidity in the diabetic population.

Recent evidence has indicated the importance of the endothelin (ET) system in the pathogenesis of diabetic complications. High glucose induces ET-1 expression in endothelial cells and the diabetic state is associated with altered ET-1 action and impaired Ca^{2+}-dependent endothelial responses (Hopfner & Gopalakrishnan, 1999). In hearts from experimental animals with STZ-induced diabetes, ET-1 receptor binding is elevated and ET-1 mRNA and protein expression are enhanced (Hileeto *et al*., 2002). The increased expression of mRNA encoding for ET-1 and ET receptors is associated with distinct myocardial injury and increased expression of extracellular matrix proteins (Chen *et al*., 2000). Hence, cardiac alterations and renal structural changes may be amenable to ET receptor antagonism (Dhein *et al*., 2000; Ding *et al*., 2003).

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The role of nitric oxide (NO) in diabetic complications is controversial. Long-term administration of the NOS substrate, L-arginine, to diabetic rats reduces the alterations in myocardial structure and function (Okruhlicova *et al*., 2002), implying that cardiac myocyte NOS is dysregulated in the course of diabetes and that the bioavailability of NO may be reduced due to oxidative stress factors (Esberg & Ren, 2003; Ye *et al*., 2003). NO also has been implicated in impaired endothelium-dependent vascular relaxation in diabetic animals and humans (Pieper, 1998). The mechanism of endothelial dysfunction may relate to impaired vascular NO synthesis or enhanced inactivation of NO by oxygen free radicals (Pieper, 1998). In addition, enhanced formation and action of ET-1 may contribute to vascular endothelial dysfunction (Kanie & Kamata, 2002). Whether in the diabetic heart myocardial ET-1 activity is augmented as a result of impaired formation or action of myocardial NO, similar to the reciprocal interaction of NO and ET-1 in endothelial cells (Warner, 1999), is not known.

In this study, we investigated the effects of chronic ET_A receptor antagonism and of endogenous NO on diabetic organ dysfunctions. In contrast to previous approaches using NO donor drugs or NOS inhibitors, which have yielded contradictory results (Smith *et al*., 1997; Joffe *et al*., 1999), we used a transgenic mouse model recently developed in our laboratory that features myocyte-specific overexpression of eNOS (Brunner *et al*., 2001). In view of recent evidence that myocyte-specific eNOS modulates *in vivo* autonomic cardiac function (Champion *et al*., 2004), this model appears ideally suited to test the importance of myocardial NO in the cardiac complications of diabetes as the transgene is permanently overexpressed specifically in the heart. In addition, it allows to differentiate between the respective roles of NO abundance (high NO levels in TG hearts) and ET_A receptor activation in diabetic cardiomyopathy. In addition, the effect of ET receptor antagonism on vascular and renal diabetic dysfunction was also studied. Diabetes mellitus was induced with STZ and ET receptors were antagonized with atrasentan, a highly potent ($K_I = 0.034$ nM) and selective (1800-fold) ET_A receptor antagonist currently in late stage clinical development for the treatment of hormone refractory prostate cancer (Nelson, 2005).

Methods

Animals and materials

TG mice over-expressing eNOS in cardiomyocytes were bred as previously described (Brunner *et al*., 2001). TG line 23 that overexpresses the eNOS gene ~60-fold (see Table 1) and WT littermates were used. All animals were derived from at least 13 back-crosses of TG mice with C57Bl/6 mice obtained from the Medical University of Vienna (Austria) and housed in a dedicated facility in approved cages. They received autoclaved food and water ad libitum. Animals aged 6–7 months and of either sex were used. Atrasentan (ABT-627) was provided by Abbott Pharmaceuticals (Abbott Park, MI, U.S.A.). All reagents were obtained from Sigma (Vienna, Austria) unless specified otherwise.

Mouse protocols

The following experimental groups were studied: (i) WT diabetic untreated ($n = 9$); (ii) TG diabetic untreated ($n = 9$); (iii) WT diabetic atrasentan-treated ($n = 9$), and i.v.) TG diabetic atrasentan-treated ($n = 8$). In order to judge the severity of pathology due to diabetes, four more groups of non-diabetic animals were used: (v) WT non-diabetic untreated; (vi) TG non-diabetic untreated; (vii) WT non-diabetic atrasentan-treated, and viii) TG non-diabetic atrasentan-treated ($n = 5$). All protocols used were in accordance with institutional guidelines.

Diabetes induction and atrasentan treatment

Type 1 diabetes was induced by a single i.p. injection of STZ (200 mg kg^{-1}) dissolved in citrate buffer (pH 4.5). Animals did not receive supplemental insulin injections and were maintained on normal chow (1324 Forti standard diet; Altromin, Lage, Germany). Atrasentan (3 mg kg^{-1} per day) was applied to diabetic groups iii, iv and non-diabetic groups vii and viii with the drinking water starting immediately after STZ treatment. The adequacy of this dose was tested by i.p. injecting 2 nmol kg^{-1} ET-1 in separate mice which raised blood pressure by 52 ± 5 mmHg (tail cuff method; TSE, Bad Homburg, Germany), while prior atrasentan treatment for 4 days completely abolished this effect ($n = 3$). Diabetes was confirmed by the presence of hyperglycaemia (see Results). After 28 days of drug dosing, non-fasted blood glucose was measured early in the morning using OneTouch Ultra glucose test strips (Lifescan, Neckargemünd, Germany), animals were heparinized (500 i.u.), anaesthetized with urethane (1 g kg^{-1}) and blood, heart, aorta and kidneys were collected and investigated on the same day (heart, aorta) or frozen (plasma, kidney) at −20°C.

Table 1 NOS catalytic activity in wild-type (WT) and eNOS transgenic (TG) nondiabetic and diabetic mice

	Nondiabetic (n = 4)		*Diabetic (n = 3)*	
	WT	*TG*	*WT*	*TG*
Total activity	0.15 ± 0.04	9.03 ± 1.64	0.19 ± 0.06	8.19 ± 3.64
L-NNA (100 μmol l^{-1})	−0.01 ± 0.01	0.1 ± 0.02	−0.01 ± 0.01	0.09 ± 0.04
1400W (10 μmol l^{-1})	0.09 ± 0.03	7.25 ± 1.30	0.11 ± 0.04	7.19 ± 3.09
EDTA (5 mmol l^{-1})	0.0 ± 0.0	−0.01 ± 0.01	0.0 ± 0.0	0.0 ± 0.0

Enzyme activity is given as pmol [^{3}H]L-citrulline formation per min and mg protein. Data are mean ± s.e.m. (for EDTA group, $n = 3$ per genotype and condition). Activities obtained in the presence of 1400 W were not significantly different from total activity (P = NS). In experiments with WT hearts, typical count rate per assay was ~250 c.p.m., in those with TG hearts, ~12,000 c.p.m. above blank count rate (~150 c.p.m.).

NOS catalytic activity

NOS catalytic activity was measured by assaying the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. Hearts ($\sim$100 mg; $n=3$–4) were harvested, rinsed in saline and homogenized in a microdismembrator (Braun, Melsungen, Germany) using 650 μl of 50 mmol l^{-1} Tris buffer (pH 7.4) containing 10 mmol l^{-1} 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5 mmol l^{-1} EDTA, 1% (v v^{-1}) 2-mercaptoethanol and 1 tablet of Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). The homogenate was subjected to three cycles of freezing and thawing, spun at 10,000 $\times g$ for 10 min at 4°C, and 20 μl of the resulting supernatant was added to 80 μl of reaction buffer containing 1 μmol l^{-1} L-arginine, $\sim$50,000 cpm [^{3}H]L-arginine (American Radiolabeled Chemicals, St Louis, MO, U.S.A.), 10 μg ml^{-1} calmodulin, 10 μmol l^{-1} tetrahydrobiopterin (Schircks Laboratories, Joan, Switzerland), 5 μmol l^{-1} flavin adenine dinucleotide, 5 μmol l^{-1} flavin mononucleotide, 200 μmol l^{-1} nicotinamide adenine dinucleotide and 500 μmol l^{-1} $CaCl_2$. Assays were incubated for 20 min at 37°C and terminated by the addition of 1 ml of stop buffer (pH 5.0) containing 2 mmol l^{-1} EDTA, 20 mmol l^{-1} sodium acetate and 1 mmol l^{-1} L-citrulline and applied to 0.5-ml columns of Dowex AG50WX-8/Na^+ followed by a wash with 1 ml distilled water. [^{3}H]L-citrulline was quantitated by liquid scintillation counting of the eluate for 10 min. Samples were combined with szintillation fluid and allowed to stand at room temperature for at least 2 h prior to counting. Control assays were done in the presence of 100 μmol l^{-1} L-NNA (NOS subtype-nonselective inhibitor), 5 mmol l^{-1} EDTA (Ca^{2+}-free condition, blocking constitutive (=endothelial + neuronal) NOS activity) or 10 μmol l^{-1} *N*-(3-(aminomethyl)benzyl)acetamidine (1400 W) (Garvey *et al*., 1997), blocking inducible NOS (iNOS) activity. The enzyme inhibitors L-NNA, 1400 W and EDTA (or distilled water in assays measuring total activity) were added to the supernatant for 15 min at 37°C prior to starting the assay.

Cardiac function

Heart perfusion experiments were performed as previously described (Brunner *et al*., 2001). Hearts were excised under anesthesia, mounted in a perfusion apparatus (Hugo Sachs Elektronik/Harvard Instruments, March-Hugstetten, Germany) and retrogradely perfused with Krebs-Henseleit buffer containing 1.25 mmol l^{-1} Ca^{2+} and 11 mmol l^{-1} glucose at 2.2 ml min^{-1} (pH 7.4, 37°C). A fluid-filled balloon ($\sim$50 μl) made of polyethylene film was inserted into the left ventricle and connected to a pressure transducer. The following cardiac parameters were monitored in unpaced hearts: left ventricular developed pressure (LVDevP; *via* a pressure transducer), left ventricular end-diastolic pressure (LVEDP; set at 5 mmHg at the beginning of the experiment), maximal rate of rise and fall of left ventricular pressure ($+dP/dt$, $-dP/dt$), coronary perfusion pressure (an index of arterial function) and heart rate (electronically derived from the pressure signal). After mounting hearts were allowed to equilibrate for 30 min, baseline parameters were recorded and the following test compounds were added in succession via sideline: norepinephrine (NE) (3–3000 nmol l^{-1}; non-cumulative dosing), followed by washout; acetylcholine (1–1000 nmol l^{-1}), followed by washout; and acetylcholine in the presence of 3 nmol l^{-1} NE (total duration of the experiment 125 min). Hearts were weighted, frozen immediately and stored at -70°C pending analysis of TBARS levels (see below).

Isometric aortic contraction and relaxation

The descending aorta was removed, cleaned and cut into 4 rings of 3–4 mm width and mounted in 5 ml-organ baths filled with oxygenated Krebs–Henseleit solution (95% oxygen, 5% carbon dioxide). Care was taken to retain the endothelium for relaxation experiments. Tissues were equilibrated for 60 min by repeatedly adjusting tension to 1.0 g and changing the bath solution. The ability of tissues to contract was tested with 30 nmol l^{-1} of the thromboxane mimetic 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U 46619). After reaching a stable contraction (30–50 min), rising concentrations of acetylcholine (0.03–1 μmol l^{-1}) were added in noncumulative fashion, with each successive dose added shortly after the maximal effect obtained with the previous dose. After washout, tissues were contracted a second time with U 46619 and the relaxation response of 100 μmol l^{-1} of the endothelium-independent relaxant diethylamine NONOate (Sampson *et al*., 2001) was tested. Tissue responses were recorded isometrically using standard force transducers.

Antioxidant effects of atrasentan

Total antioxidant status of plasma was measured with a commercial kit (Randox, Crumlin, U.K.). The assay relies on the ability of antioxidants in the plasma to inhibit oxidation of 2,2′azino-bis-[3-ethylbenz-diazoline-6-sulfonic acid] (ABTS) to $ABTS^+$ by metmyoglobin. This assay uses 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as reference. Results were expressed as Trolox equivalents (mmol l^{-1}). Levels of thiobarbituric acid reactive substances (TBARS) were measured in homogenates of heart and kidney tissues using a commercial kit (Oxitek, ZeptoMetrix Corporation, Buffalo, NY, U.S.A.). Hearts that had been used in the functional experiments as well as kidneys (after thawing) were homogenized in phosphate buffered saline (pH 7.4) and 100 μl of protein suspension ($\sim$1 mg ml^{-1}) were combined with 2.5 ml of thiobarbituric acid from the assay kit, incubated for 1 h at 95°C, cooled to room temperature and centrifuged at 1000 $\times g$. The supernatant was analyzed sprectrofluorometrically for the reaction product between thiobarbituric acid and malondialdehyde, which latter results from lipid peroxidation. TBARS levels were expressed as nmol malondialdehyde per milligram protein. The standard curve comprised 0–8 nmol ml^{-1} malondialdehyde.

Renal studies

Urine was collected over 24 h on day 27 after STZ administration using a Nalgene metabolism cage for rats. The cage was modified to minimize urine loss due to evaporation or mixing with feces. Renal protein excretion was determined using the Bradford method. Blood was collected under urethane anesthesia from the abdominal aorta at the end of day 28 and plasma ($\sim$0.45 ml) was separated and frozen. Creatinine levels in plasma and urine were measured using a validated HPLC method (Marsilio *et al*., 1999). HPLC methods give reliable values that are considerably lower than those using the Jaffé

picric acid method (Meyer *et al*., 1985). Plasma and urine samples were thawed, impurities removed and 40 μl combined with 8 μl of internal standard (=cimetidine, 25 μg ml^{-1}) and 200 μl of acetone to precipitate proteins. The samples were vortexed and kept at 4°C for 10 min before centrifugation at 13,000 r.p.m. for 5 min. The supernatant was transferred in a clean vial and evaporated in a stream of nitrogen. The sediment was reconstituted with 40 μl of 100 mmol l^{-1} NaH_2PO_4 (pH 3.0) and 20 μl aliquots were analyzed by HPLC using a LiChrospher 100 RP-18 column with 5-μm particle size (Merck, Darmstadt, Germany). The mobile phase consisted of 62% buffer (100 mmol l^{-1} NaH_2PO_4 and 30 mmol l^{-1} sodium lauryl sulfate adjusted to pH 3.0 with o-phosphoric acid) and 38% acetonitrile. The chromatographic separation was carried out at room temperature at 225 nm and took about 15 min at a flow rate of 0.8 ml min^{-1}. The concentration of creatinine was determined from an external standard regression line (4 points) without weighting.

Statistics

All values are given as mean ± s.e.m. of the number of animals or tissues indicated. Statistical analysis was performed using analysis of variance (ANOVA-2) with genotype and atrasentan treatment as factors and the parameter measured as dependent variable. If ANOVA indicated significant differences, Student's *t*-test for unpaired observations was performed. Probability values of $P<0.05$ were considered significant. Stat view (version 5.0) software on an Apple MacIntosh computer was used for analyses.

Results

Model characteristics

STZ administration resulted in similar hyperglycaemia in all experimental groups (28.7 ± 1.44, 29.6 ± 1.28, 28.6 ± 1.70 and 27.3 ± 0.94 mmol l^{-1}; P = NS); body weights were not different between the 4 diabetic groups, both at the start of experiments (combined mean: 24.6 ± 1.1 g; $n=35$) and after 4 weeks (combined mean: 21.5 ± 1.2 g; $n=35$; P = NS). In the non-diabetic groups, mean final glucose concentrations were 5.8 ± 0.28, 5.9 ± 0.17, 6.0 ± 0.44 and 6.0 ± 0.11 mmol l^{-1} ($n=5$ per group; P = NS) and body weights were 23.6 ± 1.8 g (combined mean; $n=20$) at the start of the protocol, and 24.4 ± 1.2 g after 4 weeks (combined mean; $n=20$; P = NS). Heart weights were unchanged after 4 weeks of diabetes (combined mean: 118 ± 1.7 mg; $n=35$) compared to non-diabetic animals (combined mean: 120 ± 3.5 mg; $n=20$) (P = NS). In the following heart studies, the protective actions of atrasentan on myocardial function were judged by comparing the diabetic atrasentan-treated group with the diabetic untreated group, both for the WT and TG genotype, whereas the role of NO was inferred from comparing the WT diabetic untreated group with the TG diabetic untreated group.

NOS catalytic activity

We measured NOS activity in the hearts of all groups by assaying the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in tissue homogenates (Table 1). Both WT and TG hearts contained detectable amounts of catalytic activity that is wholly blocked by N^{ω}-nitro-L-arginine (L-NNA) or in the absence of Ca^{2+} 1400 W, an iNOS-selective antagonist, had no significant effect (P = NS vs total activity). Thus, the bulk of NOS activity in hearts from non-diabetic and diabetic animals is due to the constitutive isoform.

Diabetic myocardial dysfunction – NE effects

The autonomic transmitter, NE, increased LVDevP, $+dP/dt$ and $-dP/dt$ in concentration-dependent manner (Figure 1). For all three parameters, the maximally achieved effects were lower in diabetic compared to non-diabetic hearts, reflecting diabetic cardiomyopathy. In hearts from diabetic mice treated with atrasentan, NE partially restored LVDevP (Figure 1a and b), $+dP/dt$ (Figure 1c and d) and $-dP/dt$ (Figure 1e and f) to the level observed in non-diabetic hearts. Atrasentan treatment had no effect in non-diabetic animals (Figure 1a–f). To uncover a role of NO in diabetic ventricular dysfunction, the net effects of NE were compared between the WT and TG genotypes (Figure 2). The agonist was similarly effective in WT and TG diabetic hearts (Figure 2a–c, third and fourth column), implying that local myocyte NO had no discernable effects on contraction and relaxation parameters. This was also true for non-diabetic hearts (first and second column in Figure 2a–c).

Diabetic myocardial dysfunction – acetylcholine effects

We also determined the myocardial reactivity of hearts to acetylcholine, the other autonomic agonist. In the absence of prestimulation, acetylcholine had no significant effects on systolic and diastolic function in untreated or atrasentan-treated diabetic animals (not shown). When hearts were prestimulated with 3 nmol l^{-1} NE to test for 'accentuated antagonism' of acetylcholine (Balligand, 1999), this sympathetic stimulation in part unmasked a significant improvement in basal contractile function in hearts from atrasentan-treated animals (LVDevP in WT and $-dP/dt$ in TG; see B* in abscissa of Figure 3). After pre-stimulation, acetylcholine dose-dependently reduced LVDevP (Figure 3a and b), $+dP/dt$ (Figure 3c and d) and $-dP/dt$ (Figure 3e and f). In all three cases, the concentration–effect curves were parallel, reflecting the differences in baseline values. In TG hearts (Figure 3b, d and f), STZ had no significant deleterious action on systolic function (LVDevP and $+dP/dt$), whereas it worsened diastolic function ($-dP/dt$). Accordingly, atrasentan significantly ameliorated only $-dP/dt$.

Autonomic effects on heart rate

The effects of NE and acetylcholine on heart rate are shown in Table 2. In both genotypes, NE raised heart rate and acetylcholine reduced heart rate. Similarly, following prestimulation with NE, acetylcholine reduced heart rate without significant differences between genotypes or experimental groups.

Diabetic microvascular dysfunction

Endothelium-dependent arterial relaxation is disturbed in human diabetes and in many experimental models of the

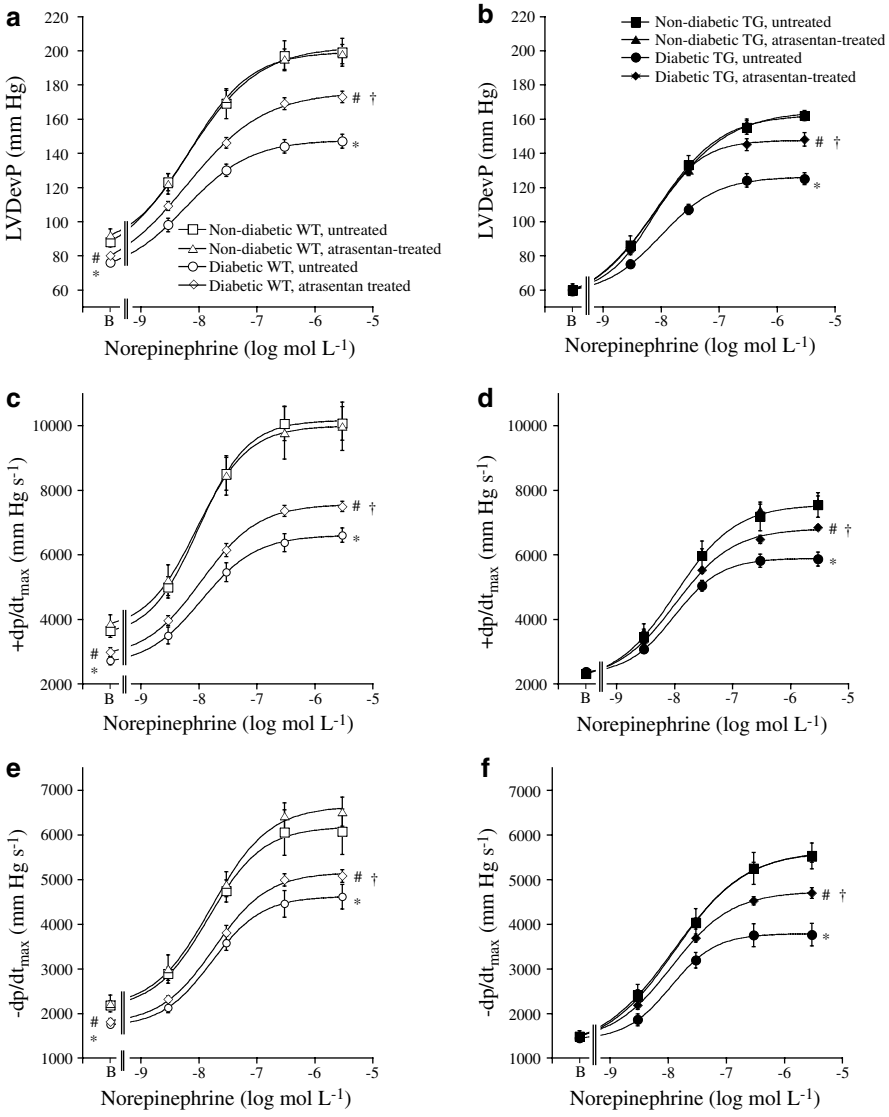

Figure 1 Myocardial effects of NE in the absence or presence of atrasentan in hearts from WT (a, c, e) or TG (b, d, f) non-diabetic and diabetic animals. Data are mean ± s.e.m. derived from five (non-diabetic) and eight to nine (diabetic) hearts. $^{*}P<0.05$ vs non-diabetic untreated group; $^{\#}P<0.05$ vs non-diabetic atrasentan-treated group; $^{\dagger}P<0.05$ vs diabetic untreated group (ANOVA). The baseline values (b) were compared by unpaired *t*-test.

disease (Pieper, 1998). We determined the relaxant effects of acetylcholine and NE which dilate coronary microvessels *via* activation of endothelial muscarinic (Brunner *et al*., 1991) and α-adrenergic receptors (Nyborg, 1990), respectively. Both agonists reduced coronary perfusion pressure, indicating coronary dilatation (Figure 4). As eNOS overexpression is restricted to myocytes, perfusion pressure reduction was similar in WT and TG hearts. Atrasentan treatment greatly improved endothelial function as evident from the much greater drop in perfusion pressure following NE (Figure 4a) or acetylcholine (Figure 4b) administration, compared to untreated diabetic hearts. Atrasentan had no effect in non-diabetic WT or TG (Figure 4a and b). When hearts from diabetic untreated or atrasentan-treated animals were prestimulated with 3 nmol l^{-1} NE, acetylcholine had no effect on coronary perfusion pressure, indicating a preferential loss of endothelium-dependent relaxation potential under adrenergic stimulation (not shown).

Diabetic macrovascular dysfunction

In organ bath experiments, constriction induced by the thromboxane mimetic U 46619 was reduced by 29 ± 3% in aortas from diabetic mice, and atrasentan completely reversed the impairment (Table 3). On the other hand, aortic relaxation induced by acetylcholine (an endothelium-dependent agonist) or diethylamine NONOate (an endothelium-independent agonist) (Sampson *et al*., 2001), were not changed by diabetes, explaining the lack of effect of atrasentan in these relaxations (Table 3).

Antioxidant effects of atrasentan

As diabetic complications are associated with exacerbated tissue oxidation, we determined plasma and tissue oxidant status using standard methods. Plasma antioxidants were quantified as Trolox equivalents in heparinized mouse plasma (Figure 5a). Plasmas from WT and TG hearts had a similar

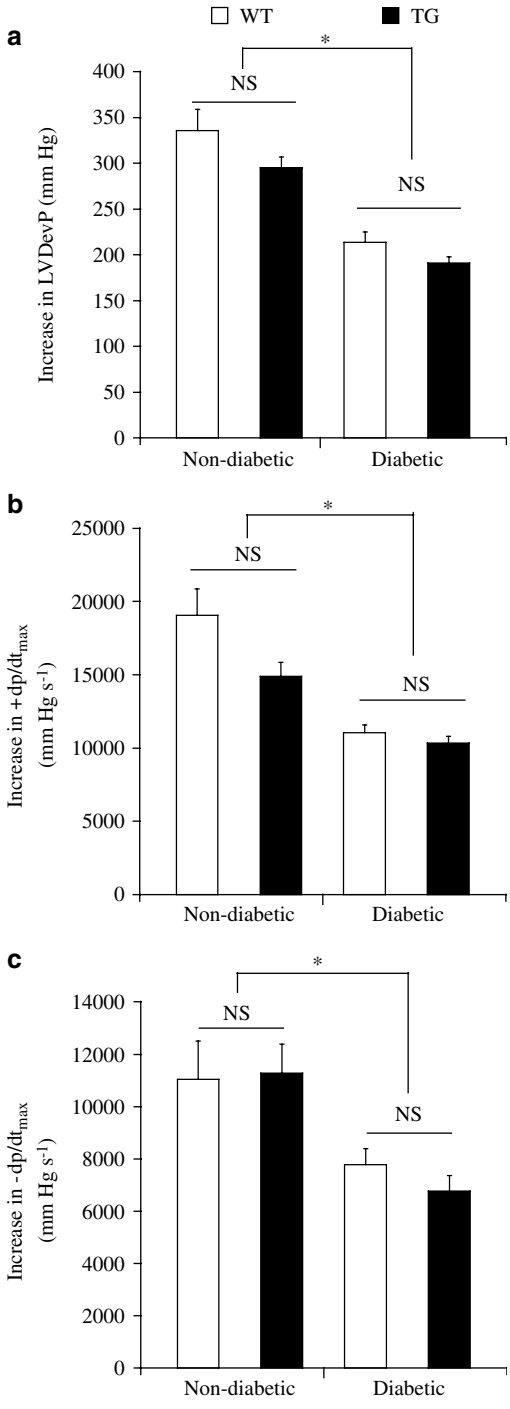

Figure 2 Net increases in LVDevP (a), $+dP/dt_{max}$ (b) and $-dP/dt_{max}$ (c) induced by NE at 3–3000 nmol l^{-1} in hearts from WT (open columns) and TG (solid columns) animals, all untreated. Data are mean ± s.e.m. derived from five (non-diabetic) and eight to nine (diabetic) hearts. $^*P<0.05$; NS = nonsignificant.

antioxidant capacity, and atrasentan treatment had no effect. This was also true for plasmas from non-diabetic animals. In contrast, tissue content of malondialdehyde equivalents, which reflects oxidant-induced lipid peroxidation reactions, was significantly increased in the heart and kidney of diabetic animals (Figure 5b and c). In both tissues, atrasentan significantly improved the oxidant status as evident from the reduction of malondialdehyde concentrations to the level observed in non-diabetic organs. As expected, there was not difference between genotypes, and in tissues from non-diabetic animals atrasentan had no effect.

Renal function

Diabetic animals produced ~six-times as much urine over 24 h as non-diabetic animals, and atrasentan significantly reduced polyuria (Figure 6a). Urinary protein excretion was about twice as high in diabetic compared to non-diabetic animals ($P<0.05$), but atrasentan had no significant effect (Figure 6b). In view of improved renal tissue oxidant status following atrasentan treatment (Figure 5c), we measured creatinine levels in plasma and urine. Plasma levels were close to 10 μmol l^{-1} (0.1 mg dl^{-1}) in all groups. Atrasentan slightly decreased creatinine in plasma ($P<0.05$), but not in urine of the diabetic group (Figure 6c and d) so that calculated creatinine clearance was not significantly augmented (Figure 6e) Thus, atrasentan had modest protective effects on renal parameters at this early stage of diabetes.

Discussion

The present study addresses the hypothesis that cardiovascular complications of type 1 diabetes are partly consequences of an activated ET system which, in conjunction with an altered NO/cGMP pathway, could lead to myocardial and vascular dysfunction. We showed that oral application of the endothelin ET_A receptor antagonist atrasentan for 4 weeks ameliorated myocardial function, microvascular coronary function, tissue oxidant status and some aspects of renal function. Unlike the ET system, myocardial NO appears to play a minor role in diabetic ventricular dysfunction, because TG hearts with eNOS overexpression behaved identically to WT hearts.

We observed substantial hyperglycaemia and a small weight loss along with cardiac dysfunction at 4 weeks after diabetes induction, both in WT and TG mice. ET-1 is strongly implicated in the observed functional aberrations because systolic myocardial contractile parameters as well as diastolic relaxation in response to adrenoceptor stimulation were consistently improved by ET_A receptor blockade. These data support the view that the contraction of cardiac myocytes in early type 1 diabetes is intrinsically abnormal (Severson, 2004) and that the aberrations can be substantially alleviated by blocking ET_A receptors. The mechanism of the beneficial effects of atrasentan on diabetic heart function is unknown at present. Under normal circumstances, ET-1 has positive inotropic and chronotropic actions on cardiac myocytes (Kelly *et al*., 1990), but under conditions of β-adrenergic stimulation ET-1 exerts a potent inhibitory effect mediated through an ET_A receptor/G_i-mediated pathway (Thomas *et al*., 1997). In agreement with the latter proposition, ET-1 is reported to inhibit cAMP formation, also *via* a G_i protein-related mechanism, in response to isoprenaline and forskolin in adult cardiac myocytes (Jones, 1996). Thus, endogenous ET-1 attenuates intrinsic cardiac responses to adrenergic activation leading to cardiac dysfunction in diabetic hearts.

On the other hand, Figure 2 clearly shows that the beneficial effects of atrasentan are of similar magnitude in WT and TG hearts, implying that supplemental myocardial NO has no

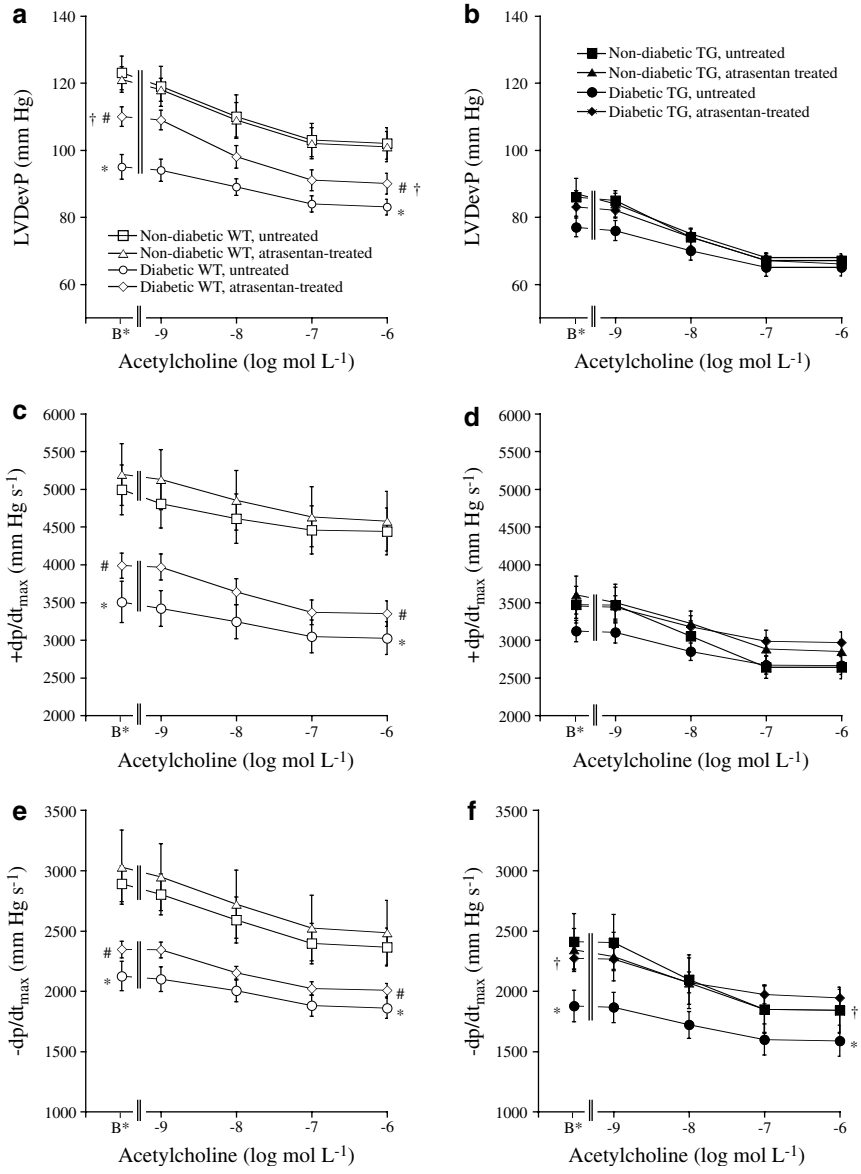

Figure 3 Myocardial effects of acetylcholine in the absence or presence of atrasentan in hearts from WT (a, c, e) or TG (b, d, f) non-diabetic and diabetic animals. Hearts were pre-stimulated with 3 nmol l^{-1} NE (indicated as B* on the abscissa). Data are mean ± s.e.m. derived from five (non-diabetic) and eight to nine (diabetic) hearts; $^{*}P<0.05$ vs non-diabetic untreated group; $^{\#}P<0.05$ vs non-diabetic atrasentan-treated group; $^{\dagger}P<0.05$ vs diabetic untreated group (ANOVA). The baseline values (B*) were compared by unpaired *t*-test.

Table 2 Effects of maximum concentrations of NE (3 μmol l^{-1}), acetylcholine (100 nmol l^{-1}) and of acetylcholine (1 μmol l^{-1}) in the presence of NE (3 nmol l^{-1}) on spontanous heart rate in WT or TG animals

	Nondiabetic untreated		*Nondiabetic atrasentan-treated*		*Diabetic untreated*		*Diabetic atrasentan-treated*	
	WT	*TG*	*WT*	*TG*	*WT*	*TG*	*WT*	*TG*
Baseline	397 ± 12	399 ± 14	392 ± 17	394 ± 5	391 ± 9	396 ± 7	387 ± 7	386 ± 8
NE	575 ± 23	568 ± 22	560 ± 8	562 ± 22	547 ± 10	568 ± 13	574 ± 10	563 ± 9
Acetylcholine	309 ± 9	320 ± 15	318 ± 18	308 ± 9	301 ± 7	299 ± 8	296 ± 5	294 ± 6
Acetylcholine + NE	373 ± 8	352 ± 20	370 ± 14	354 ± 5	387 ± 7	386 ± 6	384 ± 9	385 ± 7

Heart rates are given in beats min^{-1}. Data are mean ± s.e.m. derived from five (nondiabetic) and eight to nine (diabetic) hearts. There were no significant effects between groups. NE: norepinephrine.

discernable protective role in diabetic cardiac dysfunction. High levels of NO are known to exert negative inotropic and lusitropic actions, mainly as a result of desensitization of contractile proteins (Shah *et al*., 1994; Brunner *et al*., 2001). As a consequence, basal ventricular function was somewhat less in TG compared to WT hearts (Figure 1). However, the quantitation of the net NE effects clearly showed that the inotropic transmitter was as active in TG as in WT diabetic

hearts, suggesting NO activity is not a limiting factor in diabetic WT hearts. In view of the ~60-fold increased NO formation in myocytes of TG hearts (Table 1), these direct functional comparisons suggest that a reduction of NO formation or NO activity suggested previously (Rösen *et al*., 1998), is unlikely to cause the myocardial impairment observed in WT diabetic hearts (compared to non-diabetic hearts). Therefore, the present results do not support the notion that increasing NO availability may be a useful strategy to prevent the development of diabetic myocardial complications in insulin-deficient states.

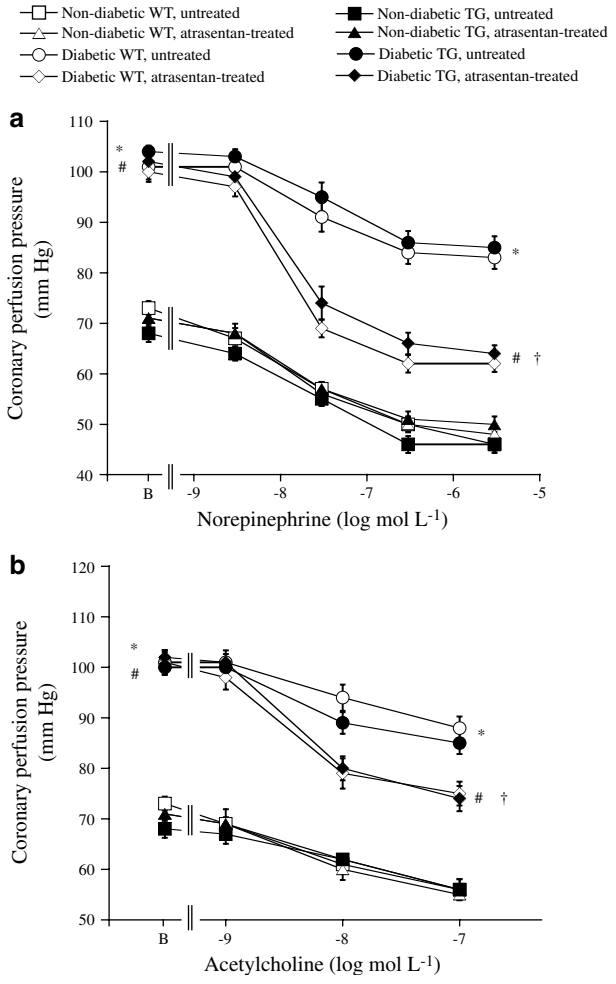

Figure 4 Coronary relaxant effects of NE (a) and acetylcholine (b) in hearts from non-diabetic and diabetic WT (open symbols) or non-diabetic and diabetic TG (solid symbols) animals either untreated (squares and circles) or treated with atrasentan (triangles and diamonds). Data are mean ± s.e.m. derived from five (non-diabetic) and eight to nine (diabetic) hearts; $^{*}P<0.05$ vs non-diabetic untreated group; $^{\#}P<0.05$ vs non-diabetic atrasentan-treated group; $^{\dagger}P<0.05$ vs diabetic untreated group (ANOVA). The baseline values (b) were compared by unpaired *t*-test. There was no difference between WT and TG groups.

Some authors have suggested that local overproduction, rather than a lack of NO plays a role in diabetic complications. A deleterious role of NO is supported by studies in myocytes exposed for 24 h to high glucose which showed functional abnormalities that were abrogated or attenuated by a NOS inhibitor, tetrahydrobiopterin, superoxide dismutase or urate, suggesting that NO, superoxide and their reaction product peroxynitrite could play a role in glucose toxicity-induced myocyte dysfunction (Esberg & Ren, 2003). The increased NO formation was attributed to enhanced expression of eNOS, but not iNOS, because protein abundance of the latter isoform was not altered. Inducible NOS was, however, implicated in diabetes-related dysfunctions in several other studies: In rats, *in vivo* inhibition of iNOS by W1400 reduced the activity of this isoform in the heart and partially restored the inotropic and coronary constrictor responses to NE at the acute phase of STZ-induced diabetes (Cheng *et al*., 2004). Neither expression nor activity of Ca^{2+}-dependent NOS was altered in these rats at 4 weeks of diabetes induction (Cheng *et al*., 2004). In STZ-induced diabetic mice the targeted deletion of the gene for iNOS improved cardiac outcome, suggesting that the presence of a functional iNOS gene during hyperglycaemia might worsen myocardial damage during ischaemia/reperfusion injury (Marfella *et al*., 2004). In the present study, 1400W had no consistent inhibitory effect on NOS catalytic activity, suggesting that the bulk of NO formed both in non-diabetic and diabetic hearts is attributable to constitutive NOS activity. Taken together, the present study does not support a cardiomyopathic role of NO at this early stage of diabetes induction.

Besides myocardial function, coronary function was significantly improved by atrasentan in the present study. It is widely accepted that the impairment of endothelium-dependent relaxation seen in diabetes may involve inactivation of NO by oxygen-derived free radicals, particularly vascular superoxide anions (Kojda & Harrison, 1999). In support, the dismutation of free radicals has generally been found to improve impaired endothelium-dependent relaxation in experimental models of diabetes mellitus (Cohen, 1993). As direct

Table 3 Aortic responses of untreated or atrasentan-treated nondiabetic and diabetic animals

	Nondiabetic untreated (n = 30)	*Nondiabetic atrasentan-treated (n = 30)*	*Diabetic untreated (n = 25)*	*Diabetic atrasentan-treated (n = 30)*
(a) Increase in aortic tone (g)				
U 46619 (30 nmol l^{-1})	0.96 ± 0.05	0.95 ± 0.03	0.68 ± 0.03*	0.93 ± 0.05†
(b) Aortic relaxation (% of precontraction)				
Acetylcholine (1 μmol l^{-1})	23 ± 2	24 ± 3	26 ± 3	25 ± 2
DEA/NO (100 μmol l^{-1})	41 ± 2	40 ± 2	41 ± 3	44 ± 2

Data are mean ± s.e.m. derived from four to five WT and three to six TG animals. Relaxation is expressed as percent of pre-contraction induced by U 46619 which amounted to 1.06 ± 0.05 g (nondiabetic untreated group), 1.00 ± 0.05 g (nondiabetic atrasentan-treated group), 0.85 ± 0.04 g (diabetic untreated) and 0.94 ± 0.04 g (diabetic atrasentan-treated), respectively. $^{*}P<0.05$ vs nondiabetic untreated group; $^{\dagger}P<0.05$ vs diabetic untreated group (*t*-test).

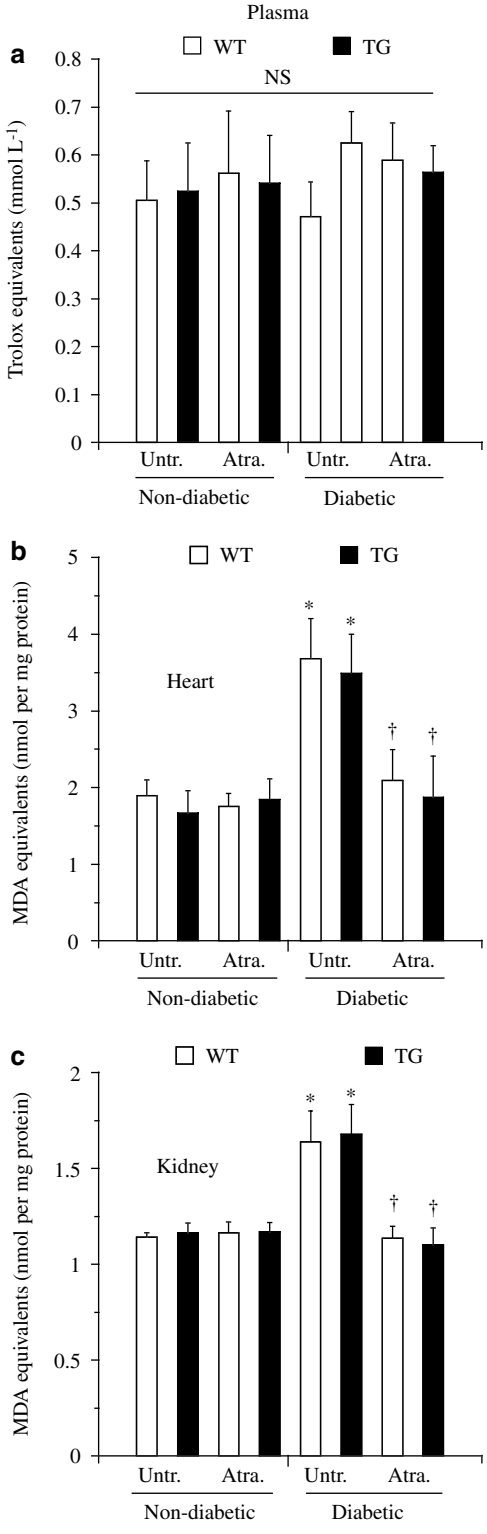

Figure 5 Antioxidant status of plasma (a), and malondialdehyde (MDA) levels in heart (b) and kidney (c). Data are mean ± s.e.m. derived from six plasmas and five (non-diabetic) and eight to nine (diabetic) organs derived from WT and TG animals, respectively. *$P<0.05$ vs non-diabetic untreated group; †$P<0.05$ vs diabetic untreated group (*t*-test). There was no difference between WT and TG groups, nor between the non-diabetic atrasentan-treated and the diabetic atrasentan-treated group. Untr. = untreated; Atra. = atrasentan-treated.

measurements of NO synthesis or release from the murine coronary endothelium are not feasible, the NO-oxidant balance within coronary endothelial cells is not known. However, in view of the antioxidant effects of atrasentan observed in cardiac homogenates (which comprise myocytes and vascular cells; Figure 5), the improved coronary relaxation (Figure 4) may be related to a reduced oxidant load within the coronary endothelium. This interpretation is in line with evidence suggesting that antioxidant defenses may be lower in diabetes (Laight *et al*., 2000). In addition, atrasentan may reduce diabetic vascular oxygen radical generation by inhibiting the ET-1-mediated activation of radical generating enzymes such as NAD(P)H oxidase and uncoupled eNOS (Hink *et al*., 2001). Thus, current evidence supports the notion that the beneficial functional effects of atrasentan may partly result from its anti-oxidant properties.

The absence of a significant effect of atrasentan on aortic relaxation (Figure 4) is not unexpected because there was no significant macrovascular impairment at 4 weeks of diabetes compared to non-diabetic animals. In fact, time of evaluation after onset of disease is critical to demonstrating macrovascular endothelial dysfunction in chemically-induced diabetes (Pieper, 1999). Recently, we made similar observations in diabetic Goto-Kakizaki rats (a model of type 2 diabetes), where an activated ET pathway contributed to elevated blood pressure, but not to vascular aortic dysfunction (Witte *et al*., 2003).

The diabetic phenotype also impaired renal function in that diabetic animals had polyuria and proteinuria, whereas creatinine clearance was not yet affected. Proteinuria is commonly observed in different nephropathies including late-stage diabetic nephropathy leading to renal failure (Ritz & Orth, 2000). Hence, slowing the progression of renal protein excretion is an accepted marker for an improvement of chronic renal disease. Atrasentan reduced 24 h urine production and plasma creatinine levels, but not renal creatinine clearance. Previous investigations have linked the ET system to renal diabetic complications on the basis of alterations in cellular ET-1 mRNA and receptor levels or favorable changes in extracellular matrix components found in diabetic animals treated with ET receptor blockers (Benigni *et al*., 1998; Dhein *et al*., 2000; Chen *et al*., 2002). In these cases, the antagonists were usually applied over 6 months or more, suggesting that ET receptor antagonists might be particularly effective in more advanced diabetic glomerulopathy.

Besides, affecting diabetic organ dysfunction, endogenous ET-1 also causes detrimental effects on glucose homeostasis that could be improved by ET antagonism. Thus, when administered to genetically obese Zucker rats (a model of insulin resistance) atrasentan reduced basal (fasting) insulin levels and the rise of plasma glucose and insulin after a meal challenge, indicating an improvement in glucose tolerance and possibly in insulin sensitivity (Berthiaume *et al*., 2005).

In conclusion, we have shown that the ET_A-selective receptor antagonist atrasentan alleviates the reduced cardiovascular performance and renal dysfunction observed in mice with STZ-induced diabetes mellitus. On the other hand, the provision of supplemental myocardial NO as a result of myocyte-specific overexpression of eNOS had no beneficial or deleterious effects on cardiac performance, implying that NO deficiency plays no role in the development of diabetic cardiomyopathy in chemically-induced diabetes.

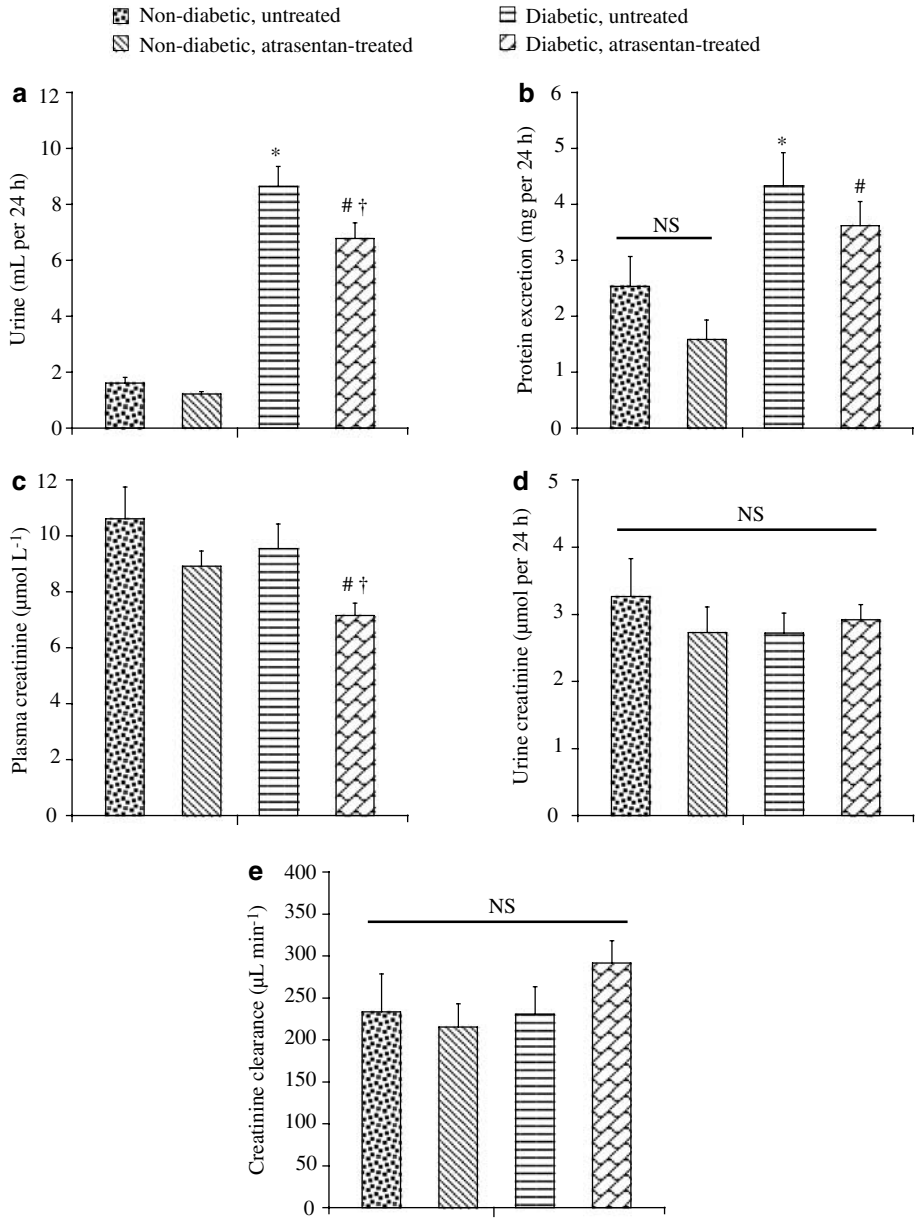

Figure 6 Effects on kidney function. The graphs show data pooled from WT and TG animals with regard to urine flow (a), proteinuria (b), plasma creatinine (c), urine creatinine (d), and renal creatinine clearance (e). Data are mean ± s.e.m. derived from 10 (non-diabetic) and 16–18 (diabetic) samples per group. $^{*}P<0.05$ vs non-diabetic untreated group; $^{\#}P<0.05$ vs non-diabetic atrasentan-treated group; $^{\dagger}P<0.05$ vs diabetic untreated group (*t*-test); NS = nonsignificant.

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References

BALLIGAND, J.-L. (1999). Regulation of cardiac β-adrenergic response by nitric oxide. *Cardiovasc. Res.,* **43,** 607–620.

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.

BERTHIAUME, N., WESSALE, J.L., OPGENORTH, T.J. & ZINKER, B.A. (2005). Metabolic responses with endothelin antagonism in a model of insulin resistance. *Metab Clin Exp,* **54,** 735–740.

BRUNNER, F., ANDREW, P., WÖLKART, G., ZECHNER, R. & MAYER, B. (2001). Myocardial contractile function and heart rate in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. *Circulation,* **104,** 3097–3102.

BRUNNER, F., KÜHBERGER, E., SCHLOOS, J. & KUKOVETZ, W.R. (1991). Characterization of muscarinic receptors of bovine coronary artery by functional and radioligand binding studies. *Eur. J. Pharmacol.,* **196,** 247–255.

CHAMPION, H.C., GEORGAKOPOULOS, D., TAKIMOTO, E., ISODA, T., WANG, Y. & KASS, D.A. (2004). Modulation of *in vivo* cardiac function by myocyte-specific nitric oxide synthase-3. *Circ. Res.,* **94,** 657–663.

CHEN, S., EVANS, T., DENG, D., CUKIERNIK, M. & CHAKRABARTI, S. (2002). Hyperhexosemia induced functional and structural changes in the kidneys: role of endothelins. *Nephron,* **90,** 86–94.

CHEN, S., EVANS, T., MUKHERJEE, K., KARMAZYN, M. & CHAKRABARTI, S. (2000). Diabetes-induced myocardial structural changes: role of endothelin-1 and its receptors. *J. Mol. Cell. Cardiol.,* **32,** 1621–1629.

CHENG, X., CHENG, X.S., KUO, K.-H. & PANG, C.Y. (2004). Inhibition of iNOS augments cardiovascular action of noradrenaline in streptozotocin-induced diabetes. *Cardiovasc. Res.,* **64,** 298–307.

COHEN, R.A. (1993). Dysfunction of vascular endothelium in diabetes mellitus. *Circulation,* **87** (Suppl V), V-67–V-76.

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.

DING, S.-S., QIU, C., HESS, P., XI, J.-F., ZHENG, N. & CLOZEL, M. (2003). Chronic endothelin receptor blockade prevents both early hyperfiltration and late overt diabetic nephropathy in the rat. *J. Cardiovasc. Pharmacol.,* **42,** 42–48.

ESBERG, L.B. & REN, J. (2003). Role of nitric oxide, tetrahydrobiopterin and peroxynitrite in glucose toxicity-associated contractile dysfunction in ventricular myocytes. *Diabetologia,* **46,** 1419–1427.

GARVEY, E.P., OPLINGER, J.A., FURFINE, E.S., KIFF, R.J., LASZLO, F., WHITTLE, B.J. & KNOWLES, R.G. (1997). 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase *in vitro* and *in vivo*. *J. Biol. Chem.,* **272,** 4959–4963.

HILEETO, D., CUKIERNIK, M., MUKHERJEE, S., EVANS, T., BARBIN, Y., DOWNEY, D., KARMAZYN, M. & CHAKRABARTI, S. (2002). Contributions of endothelin-1 and sodium hydrogen exchanger-1 in the diabetic myocardium. *Diabetes Metab Res Rev,* **18,** 386–394.

HINK, U., LI, H., MOLLNAU, H., OELZE, M., MATHEIS, E., HARTMANN, M., SKATCHKOV, M., THAISS, F., STAHL, R.A.K., WARNHOLTZ, A., MEINERTZ, T., GRIENDLING, K., HARRISON, D.G., FÖRSTERMANN, U. & MÜNZEL, T. (2001). Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ. Res.,* **88,** e14–e22.

HOPFNER, R.L. & GOPALAKRISHNAN, V. (1999). Endothelin: emerging role in diabetic vascular complications. *Diabetologia,* **42,** 1383–1394.

JOFFE, I.I., TRAVERS, K.E., PERREAULT-MICALE, C.L., HAMPTON, T., KATZ, S.E., MORGAN, J.P. & DOUGLAS, P.S. (1999). Abnormal cardiac function in the streptozotocin-induced, non-insulin-dependent diabetic rat - Noninvasive assessment with Doppler echocardiography and contribution of the nitric oxide pathway. *J. Am. Coll. Cardiol.,* **34,** 2111–2119.

JONES, L.G. (1996). Inhibition of cyclic AMP accumulation by endothelin is pertussis toxin sensitive and calcium independent in isolated adult feline cardiac myocytes. *Life Sci.,* **58,** 115–123.

KANIE, N. & KAMATA, K. (2002). Effects of chronic administration of the novel endothelin antagonist J-104132 on endothelial dysfunction in streptozotocin-induced diabetic rat. *Br. J. Pharmacol.,* **135,** 1935–1942.

KELLY, R.A., EID, H., KRÄMER, B.K., ÓNEILL, M., LIANG, B.T., REERS, M. & SMITH, T.W. (1990). Endothelin enhances the contractile responsiveness of adult rat ventricular myocytes to calcium by a pertussis toxin-sensitive pathway. *J. Clin. Invest.,* **86,** 1164–1171.

KOJDA, G. & HARRISON, D. (1999). Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc. Res.,* **43,** 562–571.

KOYA, D. & KING, G.L. (1998). Protein kinase C activation and the development of diabetic complications. *Diabetes,* **47,** 859–866.

LAIGHT, D.W., CARRIER, M.J. & ÄNGGÅRD, E.E. (2000). Antioxidants, diabetes and endothelial dysfunction. *Cardiovasc. Res.,* **47,** 457–464.

MÄKIMATTILA, S., VIRKAMÄKI, A., GROOP, P.H., COCKCROFT, J., UTRIAINEN, T., FAGERUDD, J. & YKI-JÄRVINEN, H. (1996). Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus. *Circulation,* **94,** 1276–1282.

MARFELLA, R., DI FILIPPO, C., ESPOSITO, K., NAPPO, F., PIEGARI, E., CUZZOCREA, S., BERRINO, L., ROSSI, F., GIUGLIANO, D. & D'AMICO, M.D. (2004). Absence of inducible nitric oxide synthase reduces myocardial damage during ischemia reperfusion in streptozotocin-induced hyperglycemic mice. *Diabetes,* **53,** 454–462.

MARSILIO, R., DALL'AMICO, R., GIORDANO, G., MURER, L., MONTINI, G., ROS, M., BACELLE, L., PLEBANI, M., DUSSINI, N. & ZACCHELLO, G. (1999). Rapid determination of creatinine in serum and urine by ion-pair high-performance liquid chromatography. *Int. J. Clin. Lab. Res.,* **29,** 103–109.

MEYER, M.H., MEYER JR, R.A., GRAY, R.W. & IRWIN, R.L. (1985). Picric acid methods greatly overestimate serum creatinine in mice: more accurate results with high-performance liquid chromatography. *Anal. Biochem.,* **144,** 285–290.

NELSON, J.B. (2005). Endothelin receptor antagonists. *World J. Urol.,* **23,** 19–27.

NYBORG, N.C. (1990). Action of noradrenaline on isolated proximal and distal coronary arteries of rat: selective release of endothelium-derived relaxing factor in proximal arteries. *Br. J. Pharmacol.,* **100,** 552–556.

OKRUHLICOVA, L., SOTNÍKOVA, R., STEFEK, M., TRIBULOVA, N., WEISMANN, P., GAJDOSÍK, A. & GAJDOSÍKOVA, A. (2002). L-arginine reduces structural remodeling in the diabetic rat myocardium. *Methods Find Exp. Clin. Pharmacol.,* **24,** 201–207.

PIEPER, G.M. (1998). Review of alterations in endothelial nitric oxide production in diabetes – Protective role of arginine on endothelial dysfunction. *Hypertension,* **31,** 1047–1060.

PIEPER, G.M. (1999). Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration. *Diabetologia,* **42,** 204–213.

RITZ, E. & ORTH, R. (2000). Nephropathy in patients with type 2 diabetes mellitus. *N. Engl. J. Med.,* **34,** 1127–1133.

RÖSEN, P., DU, X. & TSCHÖPE, D. (1998). Role of oxygen derived radicals for vascular dysfunction in the diabetic heart: prevention by α-tocopherol? *Mol. Cell. Biochem.,* **188,** 103–111.

SAMPSON, L.J., PLANE, F. & GARLAND, C.J. (2001). Involvement of cGMP and potassium channels in relaxation evoked by nitric oxide donor, diethylamine NONOate, in the rat small isolated mesenteric artery. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **364,** 220–225.

SEVERSON, D.L. (2004). Diabetic cardiomyopathy: recent evidence from mouse models of type 1 and type 2 diabetes. *Can. J. Physiol. Pharmacol.,* **82,** 813–823.

SHAH, A.M., SPURGEON, H.A., SOLLOTT, S.J., TALO, A. & LAKATTA, E.G. (1994). 8-Bromo-cGMP reduces the myofilament response to Ca^{2+} in intact cardiac myocytes. *Circ. Res.,* **74,** 970–978.

SHEETZ, M.J. & KING, G.L. (2002). Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA,* **288,** 2579–2588.

SMITH, J.M., PAULSON, D.J. & ROMANO, F.D. (1997). Inhibition of nitric oxide synthase by L-NAME improves ventricular performance in streptozotocin-diabetic rats. *J. Mol. Cell. Cardiol.,* **29,** 2393–2402.

TANG, W.H. & YOUNG, J.B. (2001). Cardiomyopathy and heart failure in diabetes. *Endocrinol. Metab. Clin. North Am.,* **30,** 1031–1046.

THOMAS, G.P., SIMS, S.M. & KARMAZYN, M. (1997). Differential effects of endothelin-1 on basal and isoprenaline-enhanced Ca^{2+} current in guinea-pig ventricular myocytes. *J. Physiol. (London),* **503.1,** 55–65.

WARNER, T.D. (1999). Relationships between the endothelin and nitric oxide pathways. *Clin. Exp. Pharmacol. Physiol.,* **26,** 247–252.

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.

YE, G., METREVELI, N.S., REN, J. & EPSTEIN, P.N. (2003). Metallothionein prevents diabetes-induced deficits in cardiomyocytes by inhibiting reactive oxygen species production. *Diabetes,* **52,** 777–783.

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