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RESEARCH PAPER

The phosphodiesterase-5 inhibitor vardenafil improves cardiovascular dysfunction in experimental diabetes mellitus

T Radovits[1]*, T Bömicke[1]*, G Kökény[2], R Arif[1], S Loganathan[1], K Kécsán[2], S Korkmaz[1], E Barnucz[1], P Sandner[3], M Karck[1] and G Szabó[1]

[1]*Department of Cardiac Surgery, University of Heidelberg, Heidelberg, Germany,* [2]*Department of Pathophysiology, Semmelweis University, Budapest, Hungary, and* [3]*Bayer HealthCare, Wuppertal, Germany*

Background and purpose: Patients with diabetes mellitus exhibit generalized endothelial and cardiac dysfunction with decreased nitric oxide production. Elevated intracellular cyclic guanosine monophosphate (cGMP) levels contribute to an effective cardioprotection in different pathophysiological conditions. In this study, we investigated whether chronic treatment with the phosphodiesterase-5 inhibitor vardenafil could improve diabetic cardiovascular dysfunction by up-regulating the nitric oxide–cGMP pathway in the vessel wall and myocardium.
Experimental approach: Diabetes was induced in young rats by a single intraperitoneal injection of streptozotocin (60 $mg \cdot kg^{-1}$). In the treatment group, vardenafil (10 $mg \cdot kg^{-1} \cdot day^{-1}$) was given orally for 8 weeks. Diabetic control animals received vehicle for the same time. Left ventricular pressure–volume relations were measured by using a microtip Millar pressure–volume conductance catheter, and indexes of contractility, such as the slope of end-systolic pressure–volume relationship (E_{max}) and preload recruitable stroke work (PRSW), were calculated. In organ bath experiments for isometric tension with rings of isolated aortae, endothelium-dependent and independent vasorelaxation was investigated by using acetylcholine and sodium nitroprusside.
Key results: When compared with the non-diabetic controls, diabetic rats showed increased myocardial and vascular transforming growth factor-β1 expression, impaired left ventricular contractility (impairment of E_{max} by 53%, PRSW by 40%; $P < 0.05$) and vascular dysfunction. Treatment with vardenafil resulted in higher cGMP levels, reduced transforming growth factor-β1 expression, significantly improved cardiac function (improvement of E_{max} by 95%, PRSW by 69%; $P < 0.05$) and greater vasorelaxation to acetylcholine and sodium nitroprusside in aortae from diabetic animals.
Conclusions and implications: Our results demonstrate that impaired vascular cGMP signalling contributes to the development of diabetic vascular and cardiac dysfunction, which can be prevented by chronic phosphodiesterase-5 inhibition.
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Abbreviations: DP, developed pressure; dP/dt_{max}, maximal slope of systolic pressure increment; dP/dt_{min}, maximal slope of diastolic pressure decrement; EDV, end-diastolic volume; E_{max}, slope of the left ventricular end-systolic pressure–volume relationships; eNOS, endothelial nitric oxide synthase; EIA, enzyme immunoassay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LVEDP, left ventricular end-diastolic pressure; LVSP, maximal left ventricular systolic pressure; MAP, mean arterial pressure; PDE-5, phosphodiesterase-5; PRSW, preload recruitable stroke work; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; STZ, streptozotocin; Tau, time constant of left ventricular pressure decay; TBARS, thiobarbituric acid reactive substances; TGF-β1, transforming growth factor-β1; V_p, parallel conductance volume

Introduction

Diabetes mellitus is strongly associated with cardiovascular disease. It acts as an independent risk factor for coronary atherosclerosis, but even in the absence of coronary artery disease, diabetes leads to the development of myocardial dysfunction, termed 'diabetic cardiomyopathy' (Boudina and Abel, 2007). Although, several mechanisms have

Correspondence: Tamás Radovits, Experimental Laboratory of Cardiac Surgery, Department of Cardiac Surgery, University of Heidelberg, INF 326. OG 2, 69120 Heidelberg, Germany. E-mail: radovitstamas@yahoo.com
*Equal contribution.
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been shown to be involved in the pathophysiology of diabetic cardiomyopathy (including metabolic disturbances, inflammatory processes, myocardial fibrosis, oxidative stress and endothelial dysfunction), the exact molecular mechanisms and their relative importance is still not completely understood (Fang *et al.*, 2004; Sharma and McNeill, 2006).

Under normal conditions in the coronary vasculature, endothelium-derived nitric oxide (NO) diffuses into the vascular smooth muscle, activates the soluble guanylate cyclase (sGC) enzyme resulting in increased concentration of cyclic guanosine monophosphate (cGMP), thus inducing vasorelaxation and appropriate myocardial blood supply. In contrast, reduced bioavailability of NO, down-regulation of the NO–cGMP pathway and increased formation of reactive oxygen species have been described in diabetes mellitus (Lin *et al.*, 2002; Münzel *et al.*, 2005; Haidara *et al.*, 2006).

Vardenafil is a selective inhibitor of phosphodiesterase-5 (PDE-5), an enzyme that catalyses the breakdown of cGMP, the essential second messenger involved in smooth muscle relaxation. PDE-5 inhibitors cause an accumulation of NO-driven cGMP and subsequent vasodilatation in the corpus cavernosum and in pulmonary vasculature, therefore pharmacological PDE-5 inhibition has become a widely used treatment for erectile dysfunction (Shabsigh, 2004) and pulmonary arterial hypertension (Galiè *et al.*, 2005). Effects of the different PDE-5 inhibitors on vasomotor function have also been described (Teixeira *et al.*, 2006; Schäfer *et al.*, 2008). In recent years it has been demonstrated, that not only the vascular wall, but also myocardial tissue contains PDE-5 (Giordano *et al.*, 2001; Senzaki *et al.*, 2001) and there has been considerable interest in the role of the NO–cGMP–protein kinase G (PKG) pathway in cardioprotection (Kukreja *et al.*, 2004). Recent studies reported considerable myocardial protection (Salloum *et al.*, 2006) and improvement of endothelial dysfunction (Gori *et al.*, 2005) after ischaemia/reperfusion with pharmacological PDE-5 inhibition, which were mediated by cGMP-induced opening of ATP-sensitive K^+ channels. Moreover, sildenafil, a potent PDE-5 inhibitor has been reported to improve the impaired endothelial function in smokers (Vlachopoulos *et al.*, 2004), in patients with coronary artery disease (Halcox *et al.*, 2002) and most recently in the setting of experimental diabetes (Schäfer *et al*, 2008).

In addition, intracellular cGMP accumulation has been shown to reduce oxidative tissue injury in conditions associated with increased free radical release and oxidative stress (Abdollahi *et al.*, 2003; Dias-Junior *et al.*, 2005) and even in diabetes mellitus (Milani *et al.*, 2005).

Based upon the concept of myocardial and endothelial protection by PDE-5 inhibition, we have designed experiments to test the hypothesis that pharmacological treatment with vardenafil, a highly selective and biochemically potent inhibitor of PDE-5 preserves myocardial and endothelial function in the rat model of streptozotocin (STZ)-induced experimental type 1 diabetes mellitus.

Methods

Animals

All procedures and handling of animals conformed to the *Guide for the Care and Use of Laboratory* Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). These investigations were reviewed and approved by the local Ethical Committee for Animal Experimentation.

Young adult (3 months old, 250–300 g) Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were housed in a room at a constant temperature of 22 ± 2°C with 12 h light/dark cycles and fed a standard laboratory rat diet and water *ad libitum*.

Induction of diabetes mellitus

After acclimatization, type 1 diabetes mellitus was induced in rats with a single dose of STZ at a dose of 60 $mg \cdot kg^{-1}$ intraperitoneally. STZ was freshly dissolved in citrate buffer (0.1 $mol \cdot L^{-1}$). Control animals received only the buffer. Seventy-two hours after the injection of STZ, a drop of blood was collected from the tail vein, and blood glucose concentration was determined by using a digital blood glucose metre and test strips (Accu-Chek® Sensor, Roche Inc., Mannheim, Germany). Animals with blood glucose level >15 $mmol \cdot L^{-1}$ were considered as diabetic and were included into the study.

Experimental groups, chronic treatment protocol

After confirmation of diabetes, rats were randomized to diabetic control (n = 7) and treatment (n = 8) groups. Rats injected only with citrate buffer served as non-diabetic controls (n = 8). After assignment to the study groups, animals were treated for 8 weeks with citrate buffer (0.01 $mol \cdot L^{-1}$) vehicle (non-diabetic and diabetic control groups), or with the selective PDE-5 inhibitor, vardenafil (treatment group, 10 $mg \cdot kg^{-1} \cdot day^{-1}$) in drinking water. The daily water intake was recorded, and the dose of vardenafil was accordingly adjusted.

Non-diabetic control rats (n = 25) were used for the investigation of the acute cardiac effects of vardenafil.

Haemodynamic measurements

After the treatment period, the rats were anaesthetized with a mixture of ketamine (100 $mg \cdot kg^{-1}$) and xylazine (3 $mg \cdot kg^{-1}$) intraperitoneally, tracheotomized and intubated to facilitate breathing. The animals were placed on controlled heating pads, and core temperature measured via a rectal probe was maintained at 37°C. A polyethylene catheter was inserted into the left external jugular vein for fluid administration. A 2F microtip pressure–volume catheter (SPR-838, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the ascending aorta. After stabilization for 5 min, arterial blood pressure was recorded. After that, the catheter was advanced into the left ventricle under pressure control. After stabilization for 5 min, the signals were continuously recorded at a sampling rate of 1000 s^{-1} by using

a pressure–volume conductance system (MPVS-400, Millar Instruments, Houston, TX, USA), stored and displayed on a personal computer by the PowerLab Chart5 Software System (ADInstruments Inc., Colorado Springs, CO, USA). With the help of a special pressure–volume analysis programme (PVAN, Millar Instruments, Houston, TX, USA) mean arterial pressure (MAP), maximal left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), developed pressure (DP), maximal slope of systolic pressure increment (dP/dt_{max}) and diastolic pressure decrement (dP/dt_{min}) and time constant of left ventricular pressure decay (Tau) were computed and calculated. Left ventricular pressure–volume relations were measured by transiently compressing the inferior vena cava. The slope (E_{max}) of the left ventricular end-systolic pressure–volume relationships, preload recruitable stroke work (PRSW) and dP/dt_{max}–end-diastolic volume relation (dP/dt_{max}–EDV) were calculated as load-independent indexes of left ventricular contractility.

At the end of each experiment, 100 μL hypertonic saline was injected intravenously, and from the shift of pressure–volume relations, parallel conductance volume (V_p) was calculated by the software and used for correction for the cardiac mass volume. After completing the haemodynamic measurements, blood samples were collected from the inferior caval vein for immediate determination of blood glucose (Accu-Chek® Sensor, Roche Inc., Mannheim, Germany) and for preparation of blood serum for further biochemical measurements. The volume calibration of the conductance system was performed as described previously (Pacher *et al*., 2004). Briefly, nine cylindrical holes in a block 1 cm deep and with known diameter ranging from 2 to 11 mm were filled with fresh heparinized whole rat blood. In this calibration, the linear volume-conductance regression of the absolute volume in each cylinder versus the raw signal acquired by the conductance catheter was used as the volume calibration formula.

For investigation of the acute cardiac effects of different doses of vardenafil, non-diabetic control rats received vardenafil as an intravenous bolus injection in the following doses: 0 $\mu g \cdot kg^{-1}$ ($n = 6$), 3 $\mu g \cdot kg^{-1}$ ($n = 6$), 10 $\mu g \cdot kg^{-1}$ ($n = 7$) and 30 $\mu g \cdot kg^{-1}$ ($n = 6$). We performed the above detailed haemodynamic measurements (baseline parameters and load-independent contractility indexes) 5 min after giving vardenafil.

In vitro *assessment of vascular function*

After the haemodynamic measurements, the descending thoracic aorta was carefully removed from the animals and placed in cold (+4°C) Krebs-Henseleit solution (118 $mmol \cdot L^{-1}$ NaCl, 4.7 $mmol \cdot L^{-1}$ KCl, 1.2 $mmol \cdot L^{-1}$ KH_2PO_4, 1.2 $mmol \cdot L^{-1}$ $MgSO_4$, 1.77 $mmol \cdot L^{-1}$ $CaCl_2$, 25 $mmol \cdot L^{-1}$ $NaHCO_3$, 11.4 $mmol \cdot L^{-1}$ glucose; pH = 7.4). The aortae were prepared and cleaned from periadventitial fat and surrounding connective tissue and cut transversely into rings (4 mm wide; three or four from each animal) by using an operating microscope.

Isolated aortic rings were mounted on stainless steel hooks in individual organ baths (Radnoti Glass Technology, Monrovia, CA, USA), containing 25 mL of Krebs-Henseleit solution at 37°C and aerated with 95% O_2 and 5% CO_2. Special attention was paid during the preparation to avoid damaging the endothelium.

Isometric contractions were recorded by using isometric force transducers (Radnoti Glass Technology, Monrovia, CA, USA), digitized, stored and displayed with the IOX Software System (EMKA Technologies, Paris, France).

The aortic rings were placed under a resting tension of 20 mN and equilibrated for 60 min. During this period, tension was periodically adjusted to the desired level, and the Krebs-Henseleit solution was changed every 30 min. First, potassium chloride (KCl, 100 $mmol \cdot L^{-1}$) was used in these experiments to prepare vessels for stable contractions and reproducible dose–response curves to other vasoactive agents. Maximal contraction forces to potassium chloride were determined, and aortic rings were washed until resting tension was again obtained. As different pharmacological receptor agonists widely used for pre-contraction, such as phenylephrine or the thromboxane A_2 receptor agonist U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$) elicited weak and unstable contractile responses in diabetic rats in a previous pilot study, we decided to use KCl to induce a pre-contraction before investigating vasorelaxant responses. Thus, a second KCl contraction (100 $mmol \cdot L^{-1}$) was induced until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of the endothelium-dependent dilator acetylcholine (ACh, 10^{-9}–10^{-4} $mol \cdot L^{-1}$) and the endothelium-independent dilator sodium nitroprusside (SNP, 10^{-10}–10^{-5} $mol \cdot L^{-1}$). Contractile responses are expressed as mN of tension, relaxation is expressed as percentage of contraction induced by KCl (100 $mmol \cdot L^{-1}$). pD_2 values [the negative logarithm to base 10 of the EC_{50} ($mol \cdot L^{-1}$)] were obtained from individual concentration–response curves by fitting experimental data to a sigmoidal equation by using Origin software (OriginLab, Northampton, MA, USA).

Biochemical measurements

After completing the haemodynamic measurements, urine samples were collected by puncture of the bladder, and blood samples were collected from the inferior vena cava. Blood and urine glucose were determined by a digital blood glucose metre and test strips (Accu-Chek® Sensor, Roche Inc., Mannheim, Germany). Blood samples from the inferior vena cava were collected, and serum samples were prepared and stored at –80°C.

Serum cGMP levels were determined by enzyme immunoassay (EIA) by using a commercial kit (Amersham cGMP EIA Biotrak System, GE Healthcare, Buckinghamshire, UK).

Serum thiobarbituric acid reactive substances (TBARS) as markers of lipid-peroxidation were determined by a fluorometric assay by using a commercial kit (OXItek TBARS Assay Kit, Zeptometrix Inc., Buffalo, NY, USA).

Histology and immunohistochemical analysis

Myocardial sections of the rats were removed for histological processing immediately after completing the left ventricular pressure–volume analysis. The tissue samples were fixed in

buffered paraformaldehyde solution (4%) and embedded in paraffin. Transverse sections (4 μm thick) were cut, then haematoxylin-eosin and Periodic-Acid-Schiff stainings were performed. Tissue sections were then analysed with light microscopy at a magnification of 400× for the presence of vascular wall thickening, hyalinization, bleeding or cellular degeneration. According to the method previously described (Piecha *et al.*, 2008), immunohistochemical analysis for transforming growth factor-β1 (TGF-β1) was performed on paraffin sections, by using the avidin-biotin method (anti-TGF-β1 rabbit polyclonal antibody, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and haematoxylin-counterstaining. Immunohistochemical reactivity was examined with light microscopy at a magnification of 400×. Semiquantitative scoring (scores 0–4; 0: no staining, 1: weak, 2: mild, 3: strong, 4: very strong staining) was performed as described elsewhere (Gross *et al.*, 2003).

Quantitative Real-time polymerase chain reaction (PCR)
Total RNA was isolated from whole hearts by using RNeasy Fibrous Tissue kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions. RNA concentration was determined photometrically. Reverse transcription was performed with the High Capacity cDNA Reverse Transcription kit from Applied Biosystems (Applied Biosystems, Forster City, CA, USA) by using 2 μg RNA and random primers in a final volume of 25 μL. All PCR reactions were performed on a BioRad IQ5 thermal cycler (BioRad, Budapest, Hungary) by using the Power SYBR Green PCR Master Mix (Applied Biosystems, Forster City, CA, USA). Specificity and efficiency of the PCR reaction was confirmed with melting curve and standard curve analysis respectively. Every sample was quantified in duplicate, normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Mean values are expressed with the formula $2^{-\Delta Ct}$. Primer sequences were as follows: TGF-β1 forward 5-CACCATCCATGACATGAACC-3; TGF-β1 reverse 5-TCATGTTGGACAACTGCTCC-3; endothelial nitric oxide synthase (eNOS) forward: 5-TGACCCT CACCGATACAACA-3; eNOS reverse: 5-CTGGCCTTCTGCT CATTTTC-3; GAPDH forward: 5-CAATGACCCCTTCATTGA CC-3; GAPDH reverse: 5-CGCCAGTAGACTCCACAACA-3.

Statistical analysis
All data are expressed as means ± SEM. In the case of the functional measurements and PCR experiments, inter-group comparisons were performed by using one-way analysis of variance (ANOVA) followed by Student's unpaired *t*-test with Bonferroni's correction for multiple comparisons or by the Tukey *post hoc* test. In the case of immunohistochemical analysis, Kruskal–Wallis test followed by Dunn's test was used to compare the groups. Differences were considered significant when $P < 0.05$.

Drugs
Vardenafil was provided by Bayer HealthCare (Wuppertal, Germany) and was dissolved in citrate buffer (0.01 mol·L^{-1}) (for oral application) or normal saline (for intravenous application). STZ, ACh and SNP were from Sigma-Aldrich (Taufkirchen, Germany). Drug/molecular target nomenclature conforms with BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Diabetic control rats showed lower body weight, significantly increased blood and urine glucose levels (measured after completing the haemodynamic measurements) and daily water intake. These parameters were not significantly altered by vardenafil treatment (Table 1).

Cardiac function
When compared with the non-diabetic controls, we found significantly decreased DP, dP/dt_{max}, dP/dt_{min} and increased Tau in the diabetic control group, indicating impaired systolic and diastolic function of the left ventricle. Diabetic rats treated with vardenafil showed a tendency towards higher dP/dt_{max} and dP/dt_{min} without reaching the level of statistical significance. LVSP and LVEDP did not differ in any groups studied (Figure 1).

When compared with the non-diabetic control group, diabetes mellitus in rats was associated with significantly decreased left ventricular contractility. The load-independent, PV-loop-derived contractility indexes (E_{max}, PRSW and dP/dt_{max}–EDV) showed a marked reduction in diabetic control animals (Figure 2). In the vardenafil treatment group we observed a significant increase in these parameters, indicating preserved left ventricular contractility (Figure 2). MAP did not differ in any groups studied (Table 1).

Table 1 Body weight, daily water intake, blood and urine glucose and mean arterial pressure in rats

	Control	*Diabetes*	*Diabetes + vardenafil*
Body weight (g)	448.6 ± 10.0	331.7 ± 14.2*	316.9 ± 25.1*
Daily water intake (mL)	39.7 ± 3.8	183.6 ± 17.5*	153.3 ± 13.6*
Blood glucose (mmol·L^{-1})	12.9 ± 1.7	34.7 ± 2.3*	31.2 ± 1.3*
Urine glucose (qualitative)	negative	positive	positive
Mean arterial pressure (mmHg)	77.2 ± 2.2	72.8 ± 2.5	77.6 ± 4.8

The values of body weight, daily water intake, blood and urine glucose and mean arterial pressure are shown for the different groups – control, diabetic and vardenafil-treated diabetic rats.
*$P < 0.05$ versus control.

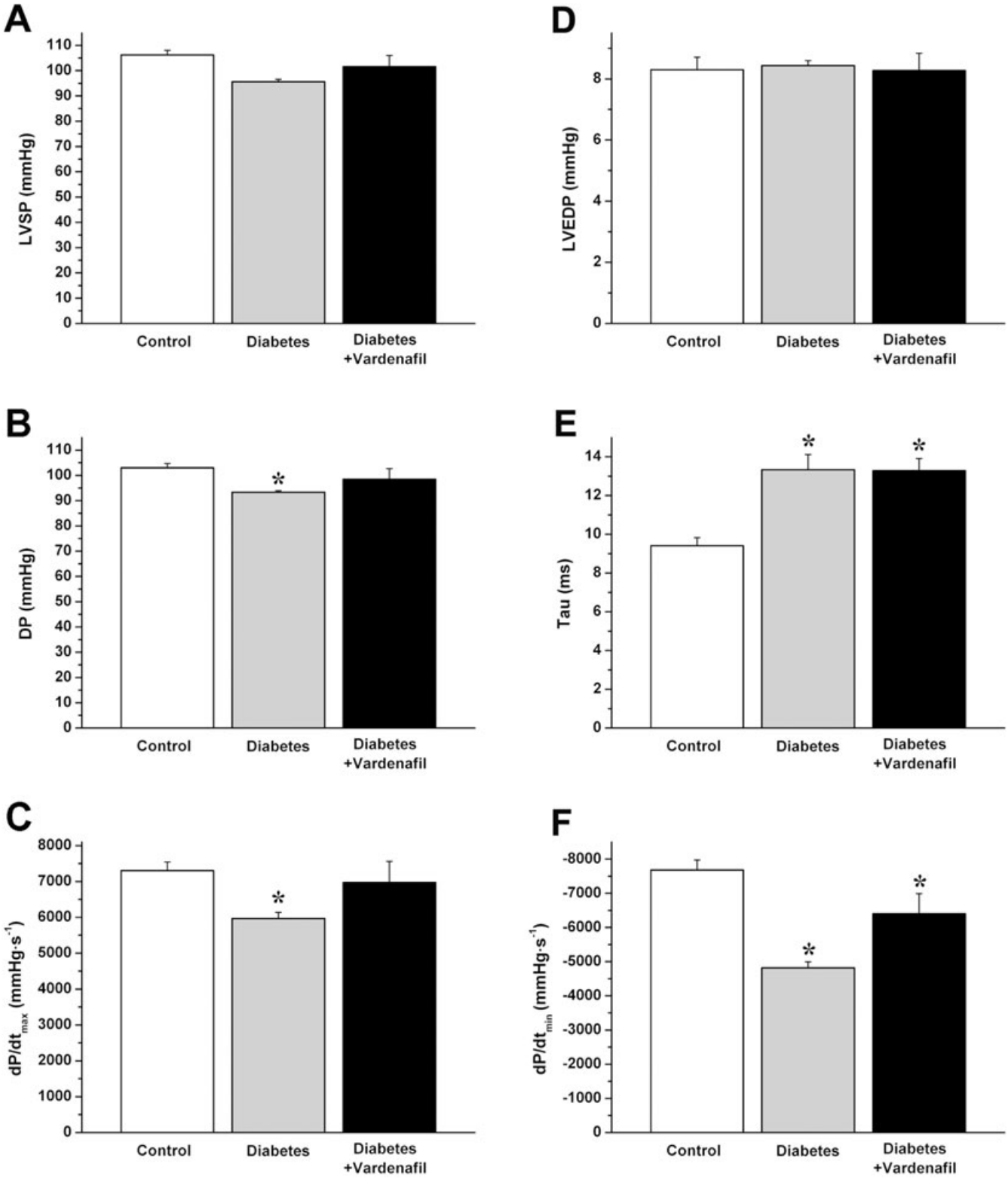

Figure 1 The effect of diabetes and vardenafil on systolic and diastolic cardiac function. Maximal left ventricular systolic pressure (LVSP) (A), developed pressure (DP) (B), maximal slope of left ventricular systolic pressure increment (dP/dt_{max}) (C), left ventricular end-diastolic pressure (LVEDP) (D), time constant of left ventricular pressure decay (Tau) (E) and maximal slope of left ventricular diastolic pressure decrement (dP/dt_{min}) (F) are shown in control, diabetic and vardenafil-treated diabetic rats. *$P < 0.05$ versus control.

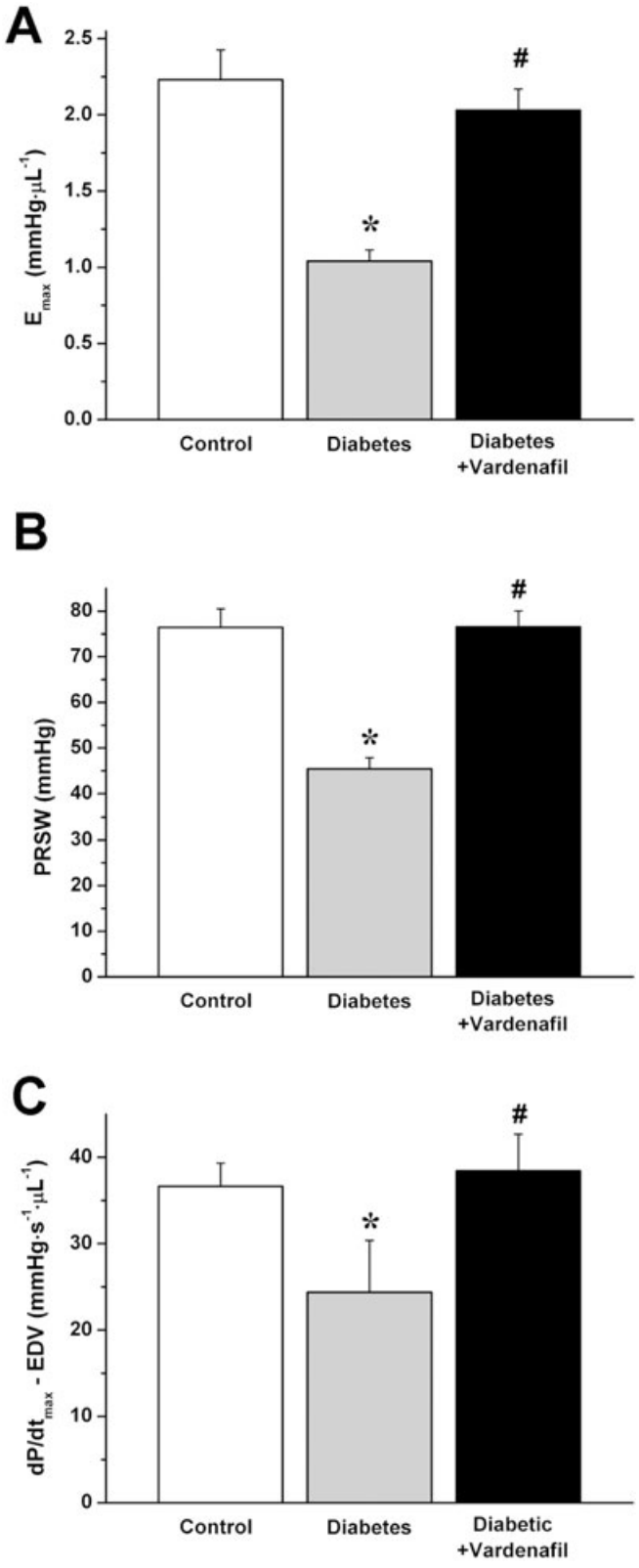

Figure 2 The effect of diabetes and vardenafil on left ventricular contractility. The slope (E_{max}) of the left ventricular end-systolic pressure–volume relationships (A), preload recruitable stroke work (PRSW) (B) and maximal slope of left ventricular systolic pressure increment–end-diastolic volume relation (dP/dt$_{max}$–EDV) (C) are shown in control, diabetic and vardenafil-treated diabetic rats. *P < 0.05 versus control; #P < 0.05 versus diabetes.

The results of our experiments on cardiac function of non-diabetic control rats treated with different doses of vardenafil showed no significant influence of the drug on left ventricular contractility as demonstrated by dP/dt$_{max}$ [7269 ± 674 vs. 7404 ± 729 vs. 7988 ± 272 vs. 7603 ± 464 mmHg·s^{-1}, in 0, 3, 10 and 30 µg·kg^{-1} vardenafil treatment group respectively, not significant (n.s.)] as well as by the load-independent contractility indexes E_{max} (2.20 ± 0.23 vs. 1.53 ± 0.32 vs. 2.10 ± 0.39 vs. 2.28 ± 0.47 mmHg·µL^{-1}, in 0, 3, 10 and 30 µg·kg^{-1} vardenafil treatment group respectively, n.s.) and dP/dt$_{max}$–EDV (30.29 ± 4.18 vs. 40.31 ± 8.27 vs. 40.55 ± 4.43 vs. 30.21 ± 6.29 mmHg s^{-1}·µL^{-1}, in 0, 3, 10 and 30 µg·kg^{-1} vardenafil treatment group respectively, n.s.).

Vascular function

A significant impairment of both the endothelium-dependent and independent vasorelaxations in diabetic control rats was demonstrated in our *in vitro* organ bath experiments after 8 weeks of diabetes. The diabetic vascular dysfunction was indicated by the reduced maximal relaxation of isolated aortic rings to ACh (P < 0.05) and SNP (P < 0.05) and the rightward shift of the concentration–response curves as compared with the non-diabetic control group. (Figure 3A,B). Treatment with vardenafil significantly improved the ACh-induced, endothelium-dependent (P < 0.05), and the SNP-induced, endothelium-independent vasorelaxations (P < 0.05) in diabetic animals (Figure 3A,B). pD$_2$ values of concentration–response curves were as follows: in the case of ACh: 6.48 ± 0.07 non-diabetic control, 6.67 ± 0.08 diabetic control, 6.66 ± 0.08 vardenafil treatment, n.s.; SNP: 7.61 ± 0.04 non-diabetic control, 7.55 ± 0.02 diabetic control, n.s.; 7.67 ± 0.03 vardenafil treatment, P < 0.05 vs. diabetic control (n = 20–29 aortic rings in each group). Maximal isometric forces produced by isolated aortic rings pre-contracted by KCl (100 mmol·L^{-1}) did not differ in any groups studied (Figure 3C).

Histology and immunohistochemical analysis

Conventional histology with haematoxylin-eosin and Periodic-Acid-Schiff staining showed moderate myodegeneration and sporadic vacuolization in the left ventricular myocardium of the diabetic control rats without signs of severe tissue damage (data not shown). Immunohistochemical staining showed increased immunoreactivity for TGF-β1 in the left ventricular myocardium (extracellular matrix and cytoplasm of cardiomyocytes) and in the wall of intracardial arteries (mainly smooth muscle cell layers) of diabetic control rats, as shown by higher TGF-β1 scores, when compared with non-diabetic control animals (Figure 4D,H). Treatment with vardenafil in diabetic rats significantly reduced myocardial TGF-β1 immunoreactivity (Figure 4). Figure 4 shows representative stainings for TGF-β1 in the left ventricular myocardium (upper panel) and in the wall of intracardial arteries (lower panel) in the different groups.

Quantitative real-time PCR

Quantitative real-time PCR from left ventricular myocardial RNA extracts revealed that mRNA expression for TGF-β1 was

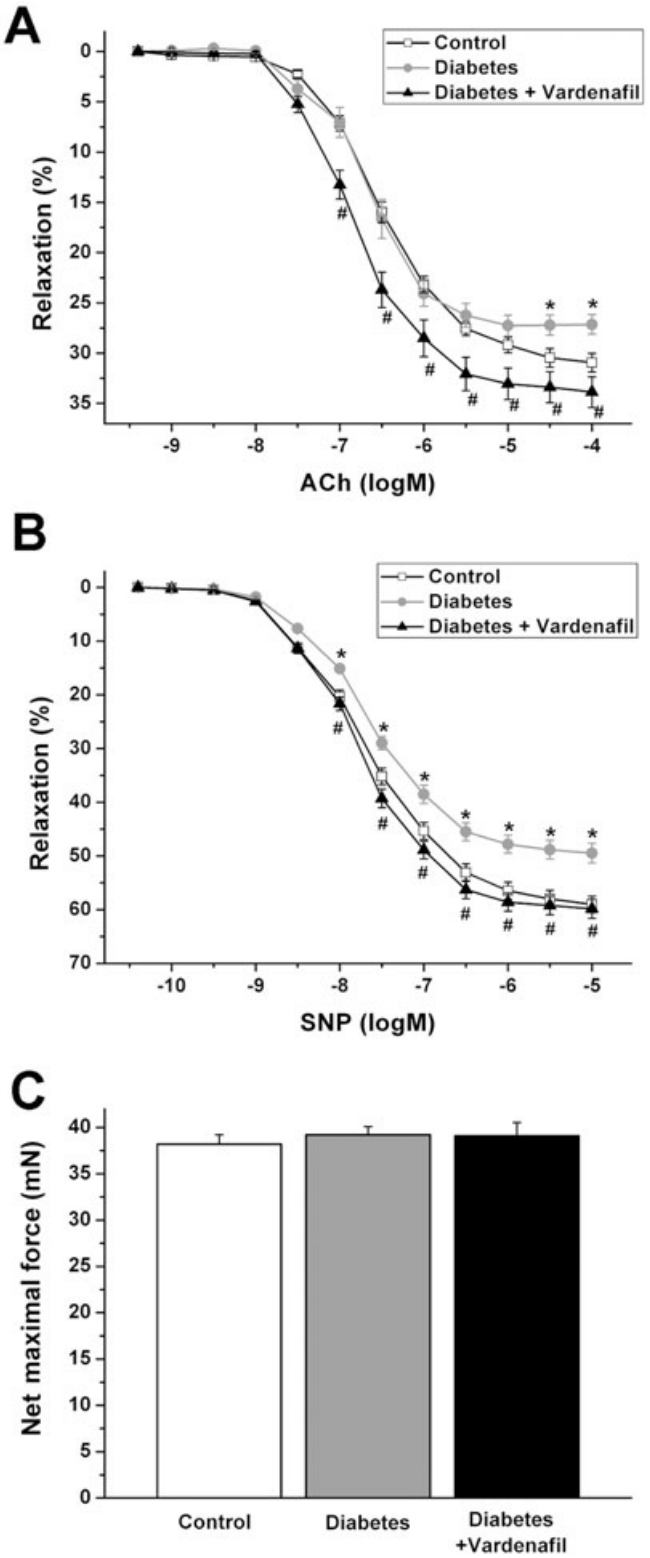

Figure 3 The effect of diabetes and vardenafil on vasomotor function of rat aortic rings. Acetylcholine (ACh)-induced endothelium-dependent vasorelaxation (A), sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation (B) and isometric contraction forces induced by potassium chloride (100 mmol·L^{-1}) (C) are shown in control, diabetic and vardenafil-treated diabetic rats. *P < 0.05 versus control; #P < 0.05 versus diabetes.

increased in diabetic control rats over non-diabetic controls. Treatment with vardenafil resulted in a significant decrease in TGF-β1 mRNA expression in diabetic rats (Figure 5A). eNOS mRNA expression was also increased in the diabetic myocardium and was not significantly altered in the vardenafil-treatment group (Figure 5B).

Biochemical measurements

Serum cGMP showed a strong tendency towards lower levels in the diabetic control group. Vardenafil treatment resulted in a dramatic increase of cGMP in the blood serum of diabetic rats (Figure 6A). Serum levels of TBARS did not significantly differ in any groups studied (Figure 6B).

Discussion

In the present study we demonstrate that pharmacological treatment with the clinically used selective PDE-5 inhibitor vardenafil in a rat model of experimental type 1 diabetes mellitus did not affect blood glucose level, but markedly increased cGMP levels, reduced myocardial and vascular TGF-β1-expression, significantly improved cardiac performance and enhanced endothelium-dependent and independent vasorelaxation. All these findings suggested improved signalling through cGMP-activated pathways.

The rat model of STZ-induced diabetes has proved useful in a wide range of research studies for the characterization of novel treatment possibilities associated with type 1 diabetes mellitus in humans. Due to its selective toxicity to the β cells of the pancreatic islets, the antibiotic STZ has been used since 1971 to create experimental diabetic animal models. Our present results on blood and urine glucose and daily water intake clearly indicate the existing pronounced diabetes mellitus in our STZ-injected rats and are compatible with results from earlier works (Litwin *et al.*, 1990; Schäfer *et al.*, 2008).

Diabetes mellitus – presumably due to persistently elevated glucose concentrations – triggers a number of mechanisms and signalling pathways that finally lead to tissue injury and functional impairment in the cardiovascular system. The exact molecular background and their pathophysiological significance are still not fully understood; however, myocardial fibrosis, oxidative stress and inflammatory pathways emerge as key mechanisms contributing to the development of diabetic cardiomyopathy and vascular dysfunction (Fang *et al.*, 2004; Sharma and McNeill, 2006). Diabetes-associated oxidative stress along with impaired NO bioavailability and chronic down-regulation of the NO–cGMP–PKG pathway have been reported in recent years (Cai and Kang, 2001; Lin *et al.*, 2002; Münzel *et al.*, 2005; Liu *et al.*, 2007), and these phenomena have been implicated in the pathogenesis of diabetic cardiovascular dysfunction.

Cyclic guanosine monophosphate, a key intracellular second messenger in cardiovascular signalling is synthetized by the sGC in response to endothelial NO. cGMP activates the cGMP-dependent protein kinase (PKG), which mediates a considerable part of the effects of cGMP, such as cell membrane hyperpolarization and vascular smooth muscle relaxation (Lincoln *et al.*, 1994). PDE enzymes hydrolyse the

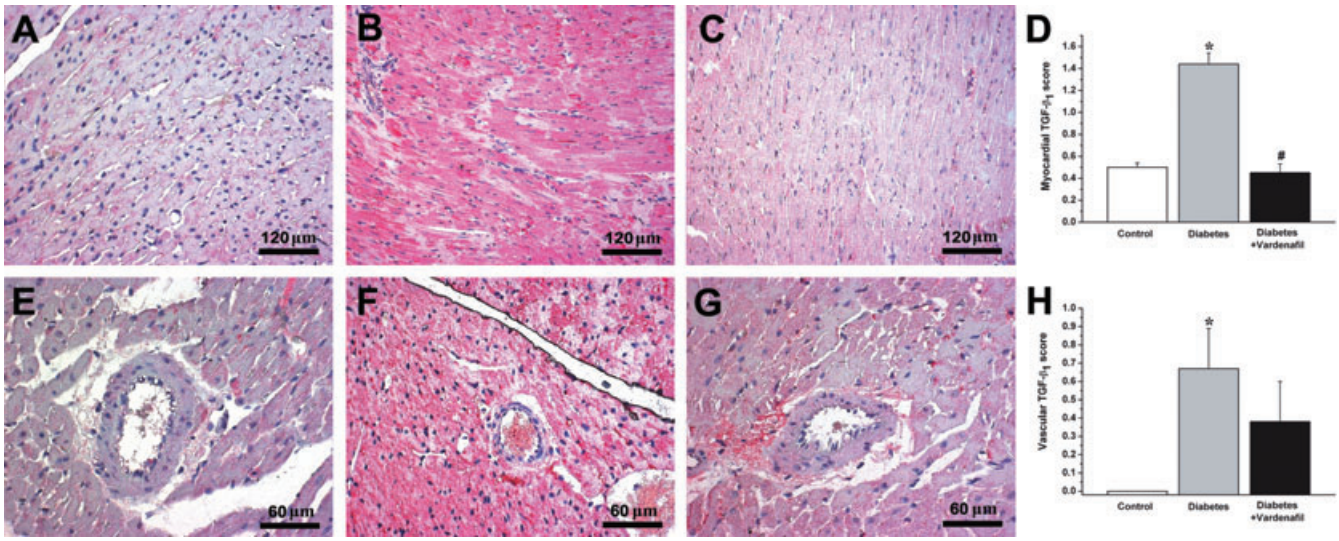

Figure 4 Representative photomicrographs and semiquantitative scoring of transforming growth factor-β1 (TGF-β1) immunohistochemistry. Representative immunohistochemical stainings for TGF-β1 (red staining) in the left ventricular myocardium (A–C) and in the wall of intracardial arteries (E–G) of control (A,E), diabetic (B,F) and vardenafil-treated diabetic (C,G) rats. Immunohistochemical scores for TGF-β1 in the left ventricular myocardium (D) and in the wall of intracardial arteries (H) in the different groups. *$P < 0.05$ versus control; #$P < 0.05$ versus diabetes. (Magnification: upper panel: 200×, lower panel 400×. Scale bar: upper panel 120 µm, lower panel: 60 µm).

phosphodiester bond of cyclic adenosine monophosphate and cGMP, thereby degrading them. PDE-5 is the predominant cGMP-metabolizing PDE, which is expressed in all vascular beds, platelets and other tissues, such as myocardium (Giordano *et al.*, 2001; Senzaki *et al.*, 2001).

It has been recently reported that elevated glucose concentrations increase the level of asymetric dimethylarginine, an endogenous inhibitor of eNOS (Lin *et al.*, 2002). Furthermore, increased oxidative stress and subsequent uncoupling of eNOS has been described in diabetes (Münzel *et al.*, 2005), both resulting in reduced NO bioavailability. The significantly increased myocardial eNOS mRNA levels in diabetic rats detected in the present study (Figure 5B) may reflect an ineffective compensatory mechanism and are in agreement with earlier results (Jesmin *et al.*, 2006). Impaired bioavailability of NO results in reduced generation of cGMP by sGC in diabetic animals (Lin *et al.*, 2002), which has been confirmed in our experiments. Another recent study showed glucose-induced reduction of PKG expression (Liu *et al.*, 2007). All these mechanisms result in a substantial down-regulation of the NO–cGMP–PKG pathway in the diabetic vessel wall and myocardium.

Inhibitors of PDE-5 block the degradation of cGMP thereby increasing its intracellular levels and evoke potent vasodilatory responses. Recently, novel cardioprotective effects of the PDE-5 inhibitor vardenafil (Salloum *et al.*, 2006) have been described and shown to be mediated by increased intracellular cGMP levels and the activation of mitochondrial ATP-sensitive K^+ channels, subsequently increased ATP synthesis and an improved energetic state of the myocardium (Salloum *et al.*, 2006). Our present results demonstrate significantly increased plasma cGMP levels (Figure 6A) in diabetic rats chronically treated with vardenafil, confirming the effectiveness of PDE-5 inhibition *in vivo* and suggesting the restoration of impaired cGMP signalling and protection of the myocardium. Considering the role of NO in nitro-oxidative stress (e.g. by peroxynitrite formation), directly increasing the levels of cGMP by PDE-5 inhibition may be more effective than triggering the pathway by NO overproduction.

The potentially antioxidative effects of PDE-5 inhibition in diabetes (Milani *et al.*, 2005) could not be confirmed in the present study. Our results on serum TBARS concentrations did not show significant differences regarding this oxidative stress marker in any groups studied (Figure 6B). Therefore, in our opinion, direct reduction of oxidative stress by PDE-5 inhibition might be a protective mechanism only of minor importance, overshadowed by the mechanism of restoring cGMP levels and thereby improving cGMP signalling in the diabetic cardiovascular system, as described above.

Numerous studies have described the importance of TGF-β1 in the context of diabetic cardiomyopathy. It plays a key role in the myocardial and vascular response to high-glucose stress in diabetes by controlling cell migration, proliferation and extracellular matrix turnover (promoting fibrosis) in the vessel wall and myocardial tissue (Agrotis and Bobik, 1996; Kanzaki *et al.*, 1997; Way *et al.*, 2002). Accordingly, our present results on myocardial and vascular TGF-β1 immunohistochemistry and on myocardial content of TGF-β1 mRNA showed markedly increased expression of this factor in diabetic rats (Figures 4 and 5A), suggesting the induction of myocardial fibrosis and vascular remodelling. In vardenafil-treated diabetic rats, a complete prevention of diabetes-induced myocardial TGF-β1 over-expression could be demonstrated at the levels of mRNA and also on the protein level (Figures 4 and 5A). These findings clearly support the concept that cGMP-activated pathways interfere with TGF-β1 signalling, presumably by PKG-mediated inhibition of TGF-β1 expression and bioactivity, as previously proposed (Wang *et al.*, 2003; Saura *et al.*, 2005). To our knowledge, this is the first report of depression of myocardial TGF-β1 signalling,

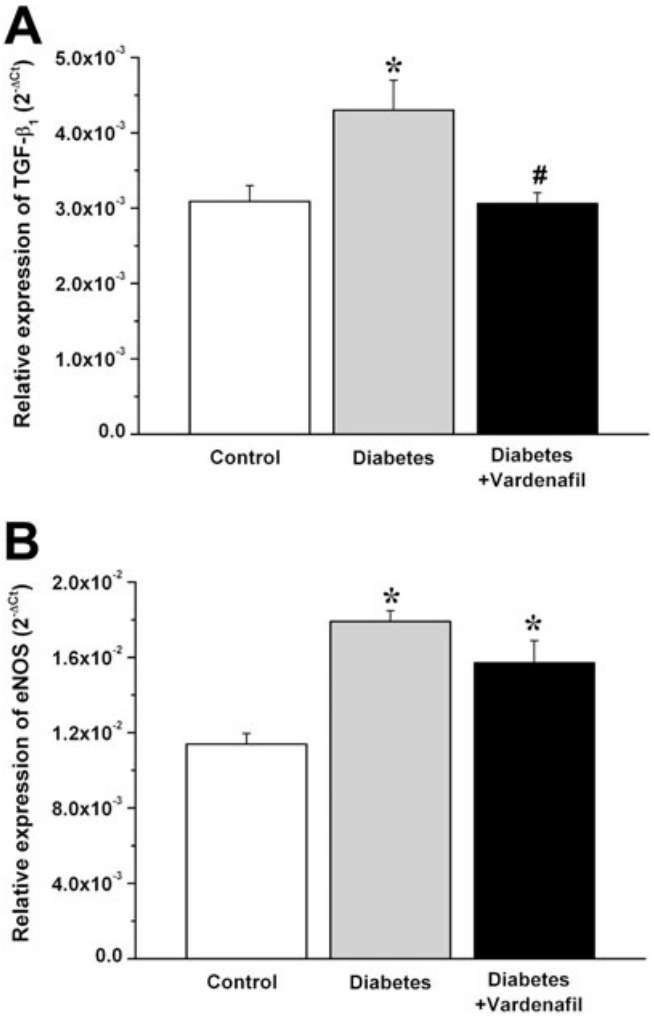

Figure 5 The effect of diabetes and vardenafil on the expression of mRNA for transforming growth factor-β1 (TGF-β1) and endothelial nitric oxide synthase (eNOS). Relative expression of mRNA for TGF-β1 (A) and eNOS (B) in the left ventricular myocardium of control, diabetic and vardenafil-treated diabetic rats. *$P < 0.05$ versus control; #$P < 0.05$ versus diabetes.

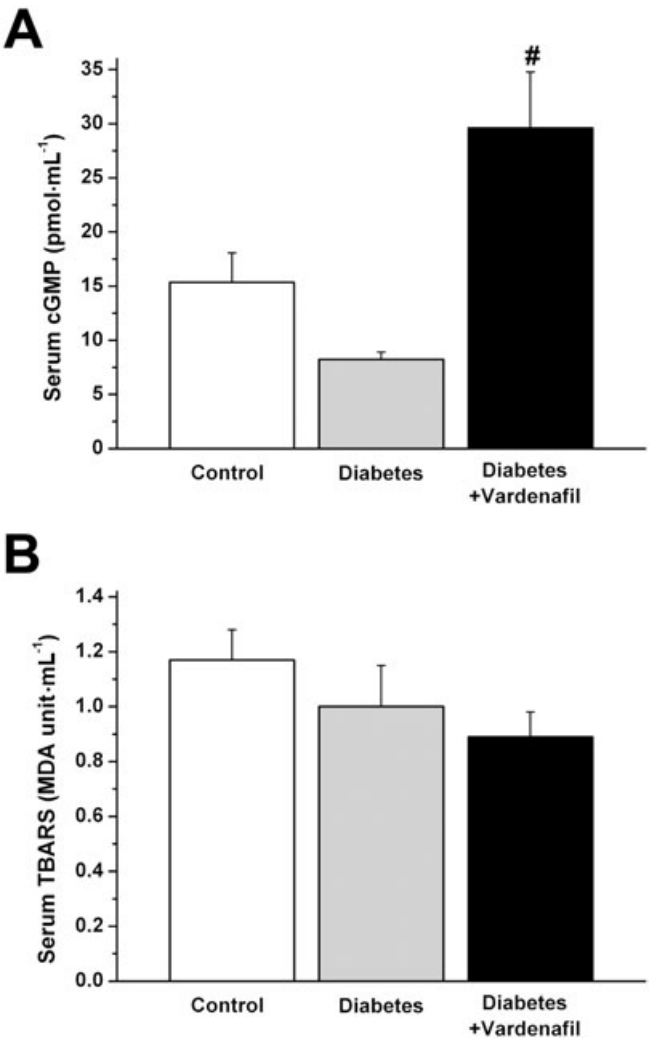

Figure 6 Serum cyclic guanosine monophosphate (cGMP) and thiobarbituric acid reactive substances (TBARS) levels. Serum concentrations of cGMP (A) and TBARS (B) in control, diabetic and vardenafil-treated diabetic rats. #$P < 0.05$ versus diabetes.

thus reducing the fibrogenic potential through PDE-5 inhibition in diabetes mellitus.

Vascular (mainly endothelial) dysfunction associated with diabetes is a well-described phenomenon (Oyama *et al.*, 1986). It is supposed to strongly correlate with erectile dysfunction (one of the current indications of PDE-5 inhibition in the clinical setting) in diabetic men. Reduced NO bioavailability and down-regulation of the NO–cGMP–PKG pathway might provide a unifying explanation (De Angelis *et al.*, 2001). Accordingly, we report here reduced serum cGMP levels along with impaired ACh-induced endothelium-dependent and the NO donor SNP-induced endothelium-independent vasorelaxation of isolated aortic rings of diabetic rats (Figure 3). Chronic treatment of diabetic rats with vardenafil resulted in higher cGMP levels and significant improvement of endothelium-dependent and independent vasorelaxation (Figure 3) indicating improved signalling through the NO–cGMP pathway. Even if the pre-contraction technique we used (KCl) might remove a part of the beneficial effects of vardenafil (by raising the extracellular K^+ concentration, the vasorelaxant effect of vardenafil through K^+ channels might be partly removed) on the vascular function of diabetic rats, we could clearly demonstrate a marked improvement of the vascular dysfunction in diabetic animals (Figure 3). This fact unequivocally confirms the favourable impact of chronic PDE-5 inhibition on vasomotor function in this model, even if a greater benefit of the treatment could have been demonstrated by using other pre-constricting agents.

Our functional data fully correspond to the results of Schäfer *et al.* (2008) using sildenafil for PDE-5 inhibition. Improvement of endothelial function and vasodilatory responses in the coronary arteries may lead to improved blood supply of the diabetic myocardium, which may secondarily ameliorate cardiac performance.

There are numerous clinical and experimental studies characterizing the cardiac functions in diabetes mellitus. Systolic and diastolic dysfunction have been described in patients and experimental animals with type 1 diabetes, by using both

non-invasive (echocardiographic) and invasive methods (Litwin *et al.*, 1990; Raev, 1994; Wichi *et al.*, 2007). Consistent with these results, we demonstrated that diabetes mellitus is associated with impaired cardiac relaxation (as reflected by prolonged Tau and by reduced dP/dt_{min}), and by depression of systolic pressure development (as indicated by decreased DP and depressed contractility index dP/dt_{max}). Although dP/dt_{max} has been used as a cardiac contractile parameter, it is well recognized, that it is load-dependent, especially on changes on preload (Kass *et al.*, 1987). That is why other PV-loop-derived indexes of left ventricular contractility have been determined in the present study. E_{max}, PRSW and dP/dt_{max}–EDV are widely used as sensitive measures of cardiac contractility, because they are independent of changes in loading conditions and therefore especially informative in assessing cardiac contractility in models, where preload and after-load are altered (Pacher *et al.*, 2004). All these load-independent indexes of left ventricular contractility, similarly to the baseline index dP/dt_{max}, were significantly decreased in diabetic control rats indicating severe contractile dysfunction (Figures 1 and 2). Without affecting MAP (Table 1) chronic treatment with vardenafil in diabetic rats moderately ameliorated the load-dependent contractility index dP/dt_{max}, while it remarkably improved all load-independent parameters of myocardial contractility (Figures 1 and 2), indicating improved contractile function. Left ventricular diastolic dysfunction in ageing animals, as indicated by dP/dt_{min} has been only slightly (not significantly) improved by PDE-5 inhibition. In our additional experiments, vardenafil did not have any direct positive inotropic effects (it did not significantly influence myocardial contractility) in control rats. Thus the improved cardiac function seen in the diabetic treatment group is a specific phenomenon, reflecting a reversal of the diabetes-associated suppressed myocardial performance, rather than the consequence of some non-specific, direct cardiac effects of vardenafil.

To our knowledge, this is the first study reporting improvement of diabetic myocardial dysfunction by pharmacological PDE-5 inhibition. As described above, the molecular mechanisms underlying these beneficial effects of vardenafil might include the up-regulation of the NO–cGMP cascade thereby inhibiting TGF-β1 signalling and thus the fibrogenic potential of diabetes. Furthermore, high cGMP levels in cardiomyocytes have been reported to inhibit PDE-3, that could lead to increased cardiac cyclic adenosine monophosphate levels (Nagendran *et al.*, 2007), and this cross-regulation of cyclic nucleotides could also contribute to the beneficial effects of PDE-5 inhibitors on cardiac function.

In summary, the current findings indicate the importance of impaired cGMP signalling and TGF-β1-activated fibrogenic pathways in the pathogenesis of diabetic myocardial and vascular dysfunction. Based on the data presented in the current report, we propose that pharmacological PDE-5 inhibition may represent a potential therapy approach to improve cardiovascular dysfunction in diabetes mellitus.

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Statement of conflict of interest

A co-author of this article, Dr Peter Sandner is an employee of Bayer HealthCare, Wuppertal, Germany.

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