Vascular Dysfunction in Streptozotocin-Induced Experimental Diabetes Strictly Depends on Insulin Deficiency

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Key Words

Streptozotocin • Diabetes • Insulin • Oxidative stress •
Endothelial dysfunction • GTP-cyclohydrolase I

Abstract

Objective: In previous studies we and others have shown that streptozotocin (STZ)-induced diabetes in rats is associated with vascular oxidative stress and dysfunction. In the present study, we sought to determine whether vascular dysfunction and oxidative stress strictly depend on insulin deficiency. **Methods:** The effects of insulin (2.5 U/day s.c., 2 weeks) therapy on vascular disorders in STZ-induced (60 mg/kg i.v., 8 weeks) diabetes mellitus (type I) were studied in Wistar rats. The contribution of NADPH oxidase to overall oxidative stress was investigated by in vivo (30 mg/kg/day s.c., 4 days) and in vitro treatment with apocynin. **Results:** Insulin therapy completely normalized blood glucose, body weight, vascular dysfunction and oxidative stress as well as increased cardiac reactive oxygen and nitrogen species formation in diabetic rats, although diabetes was already established for 6 weeks before insulin therapy was started for the last 2 weeks of the total treatment interval. Apocynin normalized cardiac NADPH oxidase activity, and L-NAME effects suggest a role for uncoupled endothelial nitric oxide synthase in dia-

betic vascular complications. **Conclusions:** Our findings indicate that STZ-induced diabetes is a model of insulin-dependent diabetes (type 1) and that cardiovascular complications are probably not associated with systemic toxic side effects of STZ.

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Introduction

Diabetes mellitus is a major risk factor for the development of cardiovascular disease [1, 2]. Endothelial dysfunction is encountered early during the development of vascular damage associated with diabetes [3]. Animal and human studies have demonstrated that increased oxidative stress largely accounts for this phenomenon and the vascular NADPH oxidase [4–6], an uncoupled endothelial nitric oxide synthase (eNOS) [5, 7, 8], xanthine oxidase [6, 9] and mitochondria [6, 10] have been identified as predominant sources of superoxide. Further support for a role of oxidative stress in diabetic vascular complica-

M.O. and M.K. contributed equally to this study and should therefore both be considered as first authors.

tions came from observations that vitamin C was able to correct endothelial dysfunction in patients with diabetes mellitus type 1 and 2 [11, 12]. eNOS uncoupling and vascular dysfunction are probably secondary to increased reactive oxygen species (ROS) formation, since ROS not only directly oxidize the eNOS cofactor BH₄ to BH₂ [8] but also trigger proteasomal degradation of the BH₄ synthesizing enzyme GCH-1 by oxidative activation of the 26S proteasome [13, 14].

Streptozotocin (STZ) is a chemotherapeutic and anti-biotic with antitumor and oncogenic properties [15, 16], which is still used today in clinical practice to treat endocrine neoplasms. Apart from that, STZ induces an inflammatory reaction of the insulin-producing β cells, resulting in insulinitis and absolute insulin deficiency. This feature accounts for its desired cytotoxic effect in the treatment of insulinomas. STZ-induced diabetes is an accepted experimental model of diabetes mellitus type 1 [17, 18], and several reports exist on STZ-induced vascular complications [19, 20]. It is well established that advanced glycation end products play an important role in the pathogenesis of STZ-induced diabetic complications [21] such as increased oxidative stress [22]. However, several reports speculated on severe systemic toxic side effects of the anticancer drug STZ [23, 24] or a different mechanism of pathogenesis of STZ-induced diabetic complications as compared to cytokine-mediated diabetes mellitus type 1 [25, 26]. Haughton et al. [27] have previously shown that insulin replacement therapy can completely normalize the adverse effects of STZ on blood glucose levels and weight gain, and Kobayashi and Kamata [28] have shown that high-dose insulin therapy (5–30 U/kg/day) can completely normalize endothelial function. In addition, Higashi et al. [29] have shown that retinal blood flow is decreased in diabetic rats and normalized by insulin therapy. It remains to be established whether in vivo treatment with a low dose of insulin rescues impaired vascular function in established diabetes.

Methods

Chemicals and Reagents

For isometric tension studies, nitroglycerin (GTN) was used from a Nitrolingual infusion solution (1 mg/ml) from G. Pohl-Boskamp (Hohenlockstedt, Germany). For induction of diabetes we used STZ from Fluka (Steinheim, Germany). L-012 [8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt] was purchased from Wako Pure Chemical Industries (Osaka, Japan). Diethylamine NONOate (DEANO) was obtained from Cayman Chemicals (Ann Arbor, Mich., USA). All other chemicals including 1,1-diphenyl-2-picrylhydrazyl free radical

(DPPH $\cdot$) and insulin (recombinant human) were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, Mo., USA), Fluka or Merck (Darmstadt, Germany).

Animals and in vivo Treatment

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the US National Institutes of Health and was granted by the Ethics Committee of the University Hospital Mainz. Male Wistar rats (6 weeks old, 250 g; Charles River Laboratories, Sulzfeld, Germany) were divided into 3 treatment groups: untreated controls (Ctr), STZ-induced diabetes mellitus type 1 treated with placebo (STZ) or insulin (2.5 U/rat/day s.c.; STZ/insulin). For induction of diabetes mellitus type 1, rats were injected with a single dose of STZ into the vena dorsalis penis (60 mg/kg i.v. in 5 mM pH 4.5 citrate buffer). Control animals were injected with the solvent. Insulin treatment was started 6 weeks after STZ injection and was administered by s.c. infusion using Alzet osmotic minipumps (Durect Corp., Cupertino, Calif., USA) with an infusion rate of 0.5 μ l/h insulin (208 U/ml) for 2 weeks. In addition, apocynin (30 mg/kg/day, for 4 days) treatment was started 7.5 weeks after STZ injection and was administered by s.c. infusion using Alzet osmotic minipumps (Durect Corp.) with an infusion rate of 1 μ l/h. After 8 weeks of total treatment duration animals were sacrificed under isoflurane anesthesia. Diabetes was diagnosed by measuring glucose levels in whole blood (for STZ-treated rats it was diluted 1:5 with NaCl solution) using the ACCU-CHEK Sensor system from Roche Diagnostics GmbH (Mannheim, Germany).

Isometric Tension Studies

Vasodilator responses to acetylcholine (ACh), GTN and DEANO were assessed with endothelium-intact isolated rat aortic rings mounted for isometric tension recordings in organ chambers, as described previously [30, 31]. Briefly, isolated aortas were cut into 4-mm segments and were mounted on force transducers (Kent, Kent, Ct., USA; Powerlab, ADInstruments Inc., Colorado Springs, Colo., USA) in organ chambers containing Krebs-Henseleit solution (37°C, pH 7.35, containing 118.3 mM NaCl, 4.69 mM KCl, 1.87 mM CaCl₂, 1.2 mM MgSO₄, 1.03 mM K₂HPO₄, 25 mM NaHCO₃, 11.1 mM D-glucose) bubbled with carbogen gas (95% O₂, 5% CO₂). To test for vasorelaxation in response to ACh, GTN and DEANO, aortic segments were stretched gradually over 1 h to reach a resting tension of 3.0 g. Following preconstriction with phenylephrine (1 μ M) to reach 50–80% of maximal tone induced by KCl, concentration-response curves to increasing concentrations of ACh, GTN and DEANO were recorded.

Detection of Oxidative Stress in Serum, Cardiac Mitochondria, Membrane Fractions and Aorta

Antioxidant capacity in serum was measured according to a previously published protocol [32]. Briefly, serum was deproteinized by addition of 50% methanol, 50 μ M DPPH $\cdot$ were added from a stock solution in DMSO, and the decrease in DPPH $\cdot$ was followed by the decreasing absorbance at 517 nm due to reduction of DPPH $\cdot$ by serum antioxidants. Isolated mitochondria were prepared from rat hearts according to a previously published protocol, and ROS formation was detected by L-012 (100 μ M)-enhanced chemiluminescence (ECL) as recently described [30, 33]. Mitochondrial suspensions were diluted to a final protein concentration of 0.1 mg/ml in 0.5 ml of PBS buffer containing L-012 (100

μM). ROS production was detected after stimulation with succinate (5 mM final concentration). The ECL was registered at intervals of 30 s over 5 min with a Lumat chemiluminometer (Berthold Technologies, Bad Wildbad, Germany), and the signal was expressed as counts/min at 5 min. Vascular ROS formation was also determined using dihydroethidine (DHE, 1 μM)-dependent fluorescence in aortic cryo-sections as reported elsewhere [34]. Membrane fractions were prepared and NADPH oxidase activity was measured by lucigenin (5 μM) ECL in the presence of NADPH (200 μM) according to a published protocol [34, 35]. On some days, the hearts of the STZ-treated rats were divided into two pieces, and one of them was incubated for 30 min at 37°C with apocynin (1 mM), then the heart pieces were homogenized, centrifuged and the membrane fractions were generated to assess the *in vitro* effect of an NADPH oxidase inhibitor on NADPH oxidase activity. Most of the apocynin was washed out during the different centrifugation steps (measured by high-performance liquid chromatography).

Determination of eNOS Uncoupling in Aorta and Oxidative Stress in Cardiac Tissue

The functional state of eNOS (coupled or uncoupled) was estimated from DHE-treated aortic cryosections in the presence and absence of the NOS inhibitor L-NAME (0.5 mM) by fluorescence microscopy as described above and as previously reported [36]. Briefly, eNOS uncoupling was assessed by densitometric quantification of DHE staining in the endothelial cell layer which was extracted from the whole microscope image. eNOS uncoupling was previously assessed by the effects of L-NAME on DHE staining [31, 34]. The method of densitometric quantification of endothelial DHE staining was adopted from the protocol of Alp et al. [37]. Cardiac oxidative stress was assessed by dot blot analysis of cardiac tissues as reported elsewhere [38]. Briefly, 100 μl (0.2 $\mu\text{g}/\mu\text{l}$ protein based on Bradford analysis) of the membranous sample was transferred to a Protran BA85 (0.45 μm) nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) by a Minifold I vacuum Dot-Blot system (Schleicher and Schuell). Each slot was washed with 250 μl PBS and the membrane was dried for 15 min at 60°C. For detection of nitrated protein, a mouse monoclonal 3-nitrotyrosine antibody (Upstate Biotechnology, Boston, Mass., USA) was used at a dilution of 1:1,000. Positive bands were detected by ECL after incubation with a peroxidase-coupled secondary antibody (GAM-POX, 1:5,000; Vector Laboratories, Burlingame, Calif., USA). All incubation and washing steps were performed according to the manufacturer's instructions. Densitometric quantification was performed by using a high-resolution scanner (Biometra/Epson) equipped with densitometry software Gel Pro Analyzer (Media Cybernetics, Bethesda, Md., USA).

Western Blot Analysis

Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. After blocking, immunoblotting was performed with antibodies against monoclonal mouse α -actinin (100 kDa) or polyclonal rabbit β -actin (42 kDa, 1:2,500; Sigma-Aldrich) as controls for loading and transfer, polyclonal goat Nox1 (1:100; Santa Cruz Biotechnologies, Santa Cruz, Calif., USA) and monoclonal mouse Nox2 (gp91^{phox}, 1:1,000; BD Biosciences, Franklin Lakes, N.J., USA), monoclonal mouse eNOS

(1:1,000; BD Biosciences), monoclonal mouse GCH-1 (1 $\mu\text{g}/\text{ml}$; Abnova Corp., Heidelberg, Germany) and monoclonal mouse dihydrofolate reductase (DHFR, 1 $\mu\text{g}/\text{ml}$; RDI Division of Fitzgerald Ind., Acton, Mass., USA). Detection was performed by ECL with peroxidase-conjugated anti-rabbit/mouse (1:10,000; Vector Laboratories) and anti-goat (1:5,000; Santa Cruz Biotechnologies) secondary antibodies. The antibody-specific bands were quantified by densitometry.

Statistical Analysis

Results are expressed as means $\pm$ SEM. One-way ANOVA (with Bonferroni's or Dunn's correction for comparison of multiple means) was used for comparisons of vasodilator potency and maximal relaxation, ROS detection by chemiluminescence or fluorescence as well as protein expression and antioxidant capacity. The EC₅₀ value for each experiment was obtained by log-transformation. $p < 0.05$ was considered significant.

Results

Blood Glucose and Weight Gain

STZ treatment of Wistar rats (STZ, single i.v. injection, 60 mg/kg, for 8 weeks) caused a 4- to 5-fold increase in blood glucose and a decrease in body weight gain within the treatment interval; both were almost completely normalized by insulin therapy (fig. 1).

Vascular Function

STZ induced endothelial dysfunction, which resulted in a significant right-shift of the ACh concentration-relaxation curve as observed by isometric tension studies (fig. 2a; potency and maximal relaxation in table 1). Therapy with insulin (2.5 U/rat/day for the last 2 weeks) completely normalized STZ-induced endothelial dysfunction (fig. 2a; table 1). Also, GTN-dependent relaxation was impaired in the setting of diabetes and was normalized by insulin co-treatment (GTN response; fig. 2b; table 1). A similar observation was made for the endothelium-independent relaxation (DEANO response; fig. 2c; table 1). *In vivo* treatment with the NADPH oxidase inhibitor apocynin significantly improved ACh potency (endothelial function) and GTN or DEANO potency (smooth muscle function), whereas less pronounced effects were observed for the maximal relaxation evoked by all of these vasodilators (table 1). Overall, apocynin *in vivo* treatment significantly improved vascular function in aortas from diabetic rats.

Vascular Oxidative Stress and eNOS Uncoupling

Aortic ROS formation was detected in cryosections using DHE-dependent fluorescent microtopography,

Fig. 1. Effects of insulin therapy on increased blood glucose levels (a) and decreased weight gain (b) in STZ-treated rats. The data are means $\pm$ SEM of 8–16 animals/group. * $p < 0.05$ vs. control group; + $p < 0.05$ vs. STZ-treated group.

Fig. 2. Effects of insulin treatment on endothelial-dependent and -independent vasodilation in STZ-treated rats. Vascular function was determined by isometric tension studies and relaxation in response to ACh (a), GTN (b) or DEANO (c). The data are means $\pm$ SEM of 26–43 aortic rings from 8–16 animals/group. * $p < 0.05$ vs. control group; + $p < 0.05$ vs. STZ-treated group.

Table 1. Effect of insulin (2.5 U/d) or apocynin (30 mg/kg/day) treatment on vascular reactivity of acetylcholine and nitroglycerin in aorta from control and streptozotocin-treated rats

Parameter	Controls ^a	STZ ^a	STZ+Ins ^a	STZ+Apo ^a
ACh potency ($pD_2 = -\log EC_{50}$) ^b	7.37 ± 0.04 (n = 40)	6.66 ± 0.05 (n = 39)*	7.24 ± 0.07 (n = 38) ⁺	7.34 ± 0.10 (n = 16) ⁺
ACh maximal relaxation, %	84 ± 2 (n = 40)	57 ± 3 (n = 39)*	85 ± 2 (n = 38) ⁺	66 ± 6 (n = 16)*
GTN potency ($pD_2 = -\log EC_{50}$) ^b	7.82 ± 0.04 (n = 43)	7.34 ± 0.06 (n = 40)*	7.70 ± 0.06 (n = 32) ⁺	7.91 ± 0.10 (n = 16) ⁺
GTN maximal relaxation, %	98 ± 0 (n = 43)	91 ± 1 (n = 40)*	98 ± 0 (n = 32) ⁺	92 ± 3 (n = 16)*
DEANO potency ($pD_2 = -\log EC_{50}$) ^b	7.10 ± 0.05 (n = 36)	6.83 ± 0.06 (n = 33)*	7.08 ± 0.05 (n = 23) ⁺	7.11 ± 0.11 (n = 16) ⁺
DEANO maximal relaxation, %	97 ± 1 (n = 36)	96 ± 1 (n = 33)	100 ± 0 (n = 23) ⁺	98 ± 1 (n = 16)

* $p < 0.05$ vs. controls; + $p < 0.05$ vs. STZ group. ^a n indicates the number of aortic rings for independent experiments. ^b EC_{50} is defined as half-maximal relaxation based on the maximal relaxation reached at the highest concentration of the vasodilator.

which was quantified by densitometry (fig. 3a, b). DHE fluorescence revealed a significant increase in ROS formation in the whole vessel wall (endothelium, media and adventitia) of vessels from STZ-treated rats, which was normalized by insulin therapy. The contribution of eNOS to overall ROS formation (uncoupling) was assessed by densitometry of the DHE fluorescence in the endothelial cell layer as well as differential effects of the NOS inhibitor L-NAME in the different groups (fig. 3c, d). ROS formation in the endothelial cell layer was increased in diabetic animals and normalized by insulin therapy. Addition of L-NAME increased the endothelial DHE signal in the control and STZ/insulin groups, whereas it decreased the signal in the endothelium in the STZ group. This is best explained by L-NAME-dependent inhibition of $\cdot\text{NO}$ formation (from intact eNOS) in the control and STZ/insulin groups. Since $\cdot\text{NO}$ is a potent superoxide scavenger (resulting in formation of peroxynitrite), inhibition of $\cdot\text{NO}$ synthesis leads to decreased breakdown of superoxide and accordingly to increased superoxide-dependent oxidation of DHE as previously reported [39]. In contrast, L-NAME-dependent inhibition of uncoupled eNOS results in decreased superoxide formation in the STZ group.

Serum Antioxidant Capacity, Mitochondrial and Nox-Derived ROS as well as Cardiac Oxidative Stress

STZ treatment caused an almost complete loss in the antioxidant capacity of serum (fig. 4a), which was measured by reduction/disappearance of the DPPH radical. STZ injection resulted in increased mitochondrial ROS formation (fig. 4b), which was detected by L-012 ECL in isolated cardiac mitochondria. Finally, STZ administration increased NADPH oxidase activity (fig. 4c), which was measured in membranous fractions by lucigenin ECL. Insulin therapy normalized all of these parameters (fig. 4). NADPH oxidase activity in hearts from diabetic animals was also normalized by in vivo or in vitro treatment with the NADPH oxidase inhibitor apocynin (fig. 4d). Cardiac oxidative stress was measured by 3-nitrotyrosine and malondialdehyde content of cardiac proteins, which was dramatically increased in the setting of diabetes and completely normalized by insulin therapy (fig. 5a, b).

Mechanisms of eNOS Uncoupling and Vascular Dysfunction in the Setting of Diabetes

eNOS expression was increased in the STZ group and normalized by insulin therapy (fig. 6a). The tetrahydrobiopterin (BH_4) synthase GTP-cyclohydrolase (GCH-1) was downregulated in the setting of diabetes (fig. 6b),

Fig. 3. Effects of insulin treatment on vascular production of ROS in STZ-treated rats. **a** DHE-fluorescent microtopography was used to assess vascular ROS formation in aortic cryosections which were incubated with DHE, and fluorescence microscopy revealed ROS formation by red staining, which was quantified by densitometry. **b** Representative microscope images. The data are means $\pm$ SEM of 16–20 aortic rings from at least 8 animals/group. * $p < 0.05$ vs. control group; + $p < 0.05$ vs. STZ-treated group. **c** The role of uncoupled eNOS for vascular ROS formation was assessed by densitometry of the DHE fluorescence in the endothelial cell layer as well as differential effects of L-NAME (500 μM) on this signal. Open columns (with L-NAME), closed columns (without L-NAME). **d** Representative microscope images; the red line indicates the border between the endothelium and the media. The data are means $\pm$ SEM of aortic rings from 5 animals/group. * $p < 0.05$ vs. control group; + $p < 0.05$ vs. STZ-treated group; ^o $p < 0.05$ vs. same group without L-NAME.

Fig. 4. Effects of insulin infusion on serum antioxidant capacity and cardiac oxidative stress in STZ-treated rats. **a** Serum antioxidant capacity was determined by tracing the reduction/disappearance of the DPPH radical by its absorption at 517 nm. **b** Cardiac mitochondrial ROS formation was measured by L-012 ECL. **c** NADPH oxidase activity in cardiac membranous fractions was detected by lucigenin ECL. **d** Effects of the NADPH oxidase inhibitor apocynin were assessed upon in vivo treatment and in vitro incubations. The data are means $\pm$ SEM of serum samples and heart tissues from 6–12 (**a–c**) or 3–4 (**d**) animals/group. * $p < 0.05$ vs. control group; + $p < 0.05$ vs. STZ-treated group.

Fig. 5. Effects of insulin therapy on cardiac content of 3-nitrotyrosine-positive (**a**) and malondialdehyde (MDA)-positive (**b**) proteins. Representative dot blots are shown at the bottom of each densitometric quantification. The data are means $\pm$ SEM of aortic rings from 5–6 animals/group. * $p < 0.05$ vs. control group; + $p < 0.05$ vs. STZ-treated group.

whereas the BH₄ 'repair' enzyme DHFR was upregulated (fig. 6c). The expression of both enzymes was normalized by insulin treatment. The increased expression of the membrane-located NADPH oxidase subunits Nox1 and Nox2 in diabetic aorta further contributed to vascular dysfunction and was normalized by insulin therapy (fig. 6d, e).

Discussion

In accordance with previous reports, we found significantly higher formation of superoxide in aortic segments from diabetic rats compared to vessels from nondiabetic animals as well as eNOS uncoupling (fig. 3). NADPH oxidase expression and activity as well as mitochondrial su-

Fig. 6. Effects of insulin therapy on vascular expression of eNOS, GCH-1, DHFR, Nox1 and Nox2 in STZ-treated rats. Expression of eNOS (a), GCH-1 (b), DHFR (c), Nox1 (d) and Nox2 (e) were assessed by using the Western blotting technique. Representative blots are shown at the bottom of each densitometric quantification. The data are means $\pm$ SEM of aortic rings from 6–12 animals/group. * $p < 0.05$ vs. control group; + $p < 0.05$ vs. STZ-treated group.

peroxide formation were increased in STZ-induced diabetes, whereas the antioxidant capacity of serum was decreased (fig. 4). In vivo treatment with the NADPH oxidase inhibitor apocynin normalized membranous ROS formation and endothelial function in tissues from diabetic rats. Downregulation of GCH-1, important for the synthesis of BH₄, was identified as a possible reason for eNOS uncoupling and vascular dysfunction in the setting of diabetes (fig. 6). The reduction of oxidative stress in vascular tissue of diabetic animals by insulin was accompanied by a restoration of endothelial function (ACh) and of the vascular smooth muscle nitrate (GTN) and •NO (DEANO) sensitivity (fig. 2). Although the latter finding is, at first view, at variance with previous data of Kobayashi and Kamata [28], it may be related to the use of DEANO in our study and of sodium nitroprusside in the other study. DEANO tends to release •NO outside the tissue, and the diffusing •NO may be trapped by ROS on its way to the media. In contrast, sodium nitroprusside needs bioactivation, and the •NO complex may reach the

media prior to bioactivation, reducing the diffusion distance and thereby the chance to be inactivated by ROS. With the endothelium-independent vasodilator GTN we frequently observed a loss of potency in aorta from diabetic or hypertensive rats; meanwhile we know that this ‘tolerance’ is due to oxidative inactivation of the redox-sensitive GTN bioactivating enzyme mitochondrial aldehyde dehydrogenase (ALDH-2) [40].

Oxidative stress is a major cause of reduced vascular •NO bioavailability in diabetes mellitus [5]. Besides the direct inactivation of •NO by reaction with superoxide [41], the resulting peroxynitrite is known to potently oxidize and thereby deplete the eNOS cofactor BH₄ [8, 42], which in turn will cause eNOS uncoupling. The first direct proof for an important role of BH₄ depletion in the vascular pathogenesis in diabetes was based on the highly protective effect of endothelium-specific overexpression of GCH-1 on diabetic complications in transgenic mice [37]. Even ex vivo adenoviral GCH-1 gene transfer was able to reverse BH₄ deficiency, repair the ability of

endothelial cells to produce $\cdot\text{NO}$ and normalize vascular function of isolated aortic vessel segments from diabetic animals [43]. Recently, we were able to show that HMG-CoA-reductase inhibition by atorvastatin treatment recouples the dysfunctional eNOS in diabetic rats by normalizing GCH-1 expression, vascular BH_4 levels, oxidative stress and endothelial function [44]. In a subsequent study we demonstrated that all superoxide sources in diabetic animals are suppressed by the AT_1 receptor blocker telmisartan and that eNOS activity as well as vascular function are normalized by telmisartan therapy [31], which was supported by findings of an independent laboratory [45].

The results of the present studies go along with this concept. As shown before [31, 46], eNOS expression was increased in the STZ group, suggesting that this may be a compensatory or 'rescue' mechanism of the organism to respond to endothelial dysfunction (fig. 6a). However, since eNOS is in an uncoupled state in diabetic animals [31, 46], a further increase in eNOS rather results in a vicious cycle. In accordance with previous observations, GCH-1, the pacemaker enzyme of BH_4 synthesis, was downregulated in the setting of diabetes, whereas the most important enzyme for BH_4 recovery, the DHFR, was upregulated (fig. 6b, c). This important finding may be the most reasonable explanation for uncoupling of eNOS, since downregulation of GCH-1 results in decreased BH_4 levels and vascular dysfunction [47]. The increased expression of the BH_4 recycling enzyme, DHFR, points to the attempt of the organism to compensate for the loss of BH_4 de novo synthesis. However, this so-called 'salvage' pathway obviously failed to recouple eNOS. It is an interesting result that 2 weeks of insulin therapy completely normalized all of these adverse effects of already established diabetes (it should be noted that STZ was injected and diabetes established 6 weeks before insulin therapy was started).

The mechanism of how GCH-1 expression is decreased in diabetes is best explained by the findings of Xu et al. [13], which demonstrate that 26S proteasome-dependent GCH-1 degradation is increased in diabetes and that inhibition of the 26S proteasome by MG132 or PR-11 prevents GCH-1 degradation and preserves its activity in diabetes mellitus. These authors also demonstrated that exposure of human umbilical vein endothelial cells to exogenous peroxynitrite increased proteasome activity and 3-nitrotyrosine in 26S proteasome.

The most likely explanation on the beneficial effect of insulin may be the inhibition of the advanced glycation end product-triggered activation and expression of vas-

cular NADPH oxidases [48], ultimately leading to increased vascular peroxynitrite production and subsequently eNOS uncoupling [5]. We here provide good evidence for an uncoupled eNOS in experimental diabetes (fig. 3c, d). Thus, the 'kindling radical concept', which implies that, for example, NADPH oxidase-mediated superoxide production results in increased peroxynitrite formation, which leads to eNOS uncoupling and subsequently to the formation of 'bonfire radicals' via an uncoupled eNOS [49], can be extended from the NADPH oxidase to xanthine oxidase and also mitochondria in the setting of diabetes. A role for NADPH oxidase in diabetic vascular complications was supported by beneficial effects of the NADPH oxidase inhibitor apocynin (fig. 4d; table 1).

Despite the fact that STZ-induced diabetes is an accepted experimental model of diabetes type 1-associated vascular dysfunction and oxidative stress [19, 20], several reports speculated on severe systemic toxic side effects of the anticancer drug STZ [23, 24]. With the present study we demonstrate that STZ-induced diabetes is a model of insulin-dependent diabetes (type 1) and that STZ-dependent vascular complications are probably not associated with systemic toxic side effects but strictly depend on insulin deficiency. These findings are in good agreement with previous reports on insulin replacement therapy, in which insulin completely normalized the adverse effects of STZ on vascular responsiveness and vascular superoxide formation [28], on blood glucose levels and weight gain [27] as well as on STZ-triggered vasoconstrictive responses to both endothelin-1 and methoxamine [50]. More support for our concept came from Higashi et al. [29], who reported that retinal blood flow is decreased in diabetic rats and normalized by insulin therapy, although these authors used a different protocol for induction of diabetes and found that vascular abnormalities are not completely reversed by short-term insulin therapy upon long-term untreated diabetes. However, our results are at variance with previous data on insulin therapy failing to improve pulmonary vascular complications in STZ rats [51]. It should be noted that the use of the thoracic aorta as a nitric oxide-sensitive contractile vessel in the present study represents a limitation since it hardly reflects the situation in the microvascular system. Although we did not assess data on cardiac function and therefore cannot speak of cardiovascular complications in our study, we quantified markers of oxidative stress such as 3-nitrotyrosine and malondialdehyde in cardiac tissue. These increased levels of oxidatively modified proteins suggest adverse effects of STZ-induced hyperglycemia on cardiac

function. We know from previous studies that prostacyclin synthase is nitrated and inactivated in the setting of diabetes, removing another important vasodilator under hyperglycemic conditions [52, 53]. We conclude that STZ-induced diabetes mellitus is a valid model of hyperglycemia because the major vascular side effects of diabetes like vascular dysfunction and oxidative stress can be delineated to insulin deficiency and are not due to unspecific toxic side effects of STZ on the vasculature.

Acknowledgments

We thank Jörg Schreiner and Sebastian Steven for expert technical assistance. The present work was supported by generous financial support by the Johannes Gutenberg University and Medical Center Mainz (MAIFOR and Forschungsfonds grants to A.D.). M.K. holds a stipend from the 'Mainzer Herz Stiftung'. This paper contains results that are part of the doctoral thesis of Tjebo Heeren.

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