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Protection against oxidative stress in diabetic rats: Role of angiotensin AT_1 receptor and beta 1-adrenoceptor antagonism

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Abstract

Oxidative stress and low-grade inflammation are hallmarks of diabetes mellitus. We explored protective, blood pressure-independent effects of the angiotensin II type 1 (AT_1) receptor antagonist candesartan and the selective β_1-adrenoceptor antagonist metoprolol. Diabetes mellitus was induced in 8-week-old Sprague–Dawley rats after injection of streptozotocin. Diabetic rats were randomized to treatment with candesartan or metoprolol in sub-antihypertensive doses or to placebo treatment. In the quadriceps, musculature markers of oxidative stress and inflammation were determined. Function of the inherent vascular bed was measured in vivo in the autoperfused hindlimb. Increases in NAD(P)H activity, expression of its cytosolic subunit $p22^{phox}$ and of endothelial NO synthase e(NOS) displayed enhanced oxidative stress. Upregulated intercellular (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 and of inducible NOS (iNOS) revealed inflammatory processes. Diabetes was associated with severe impairment of endothelium-dependent and -independent vasodilatation. Candesartan, but not metoprolol, reduced NAD(P)H activity, attenuated diabetes-induced over-expression of $p22^{phox}$ and eNOS mRNA as well as ICAM-1, VCAM-1, iNOS and eNOS immunoreactivity and led to a substantial improvement of endothelium-dependent vasodilatation (+46.3% vs. placebo treatment; $P<0.05$). Angiotensin AT_1 receptor antagonism, but not β_1-adrenoceptor antagonism, ameliorates diabetes-generated oxidative stress, indicating a pivotal role of the renin–angiotensin system in the development of diabetic complications.

Keywords: Diabetes mellitus; Endothelial dysfunction; NAD(P)H oxidase; Oxygen radical; Beta 1-adrenoceptor antagonist; Angiotensin AT_1 receptor antagonist

1. Introduction

There is definite evidence that reactive oxygen species contribute to the progression of diabetes mellitus, its complications, associated cardiovascular disease and atherosclerosis (Evans et al., 2002). Enhanced oxidative stress reduces availability of nitric oxide (NO) and promotes redox-sensitive expression of adhesion molecules like intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Hsueh and Quinones, 2003). These cell surface glycoproteins facilitate the attachment of blood-borne leukocytes to the endothelium and are crucial for the resulting low-grade inflammation (Harrison, 1997). Angiotensin II accelerates the development of diabetic complications by promoting the generation of superoxide anions by activation of the angiotensin AT_1 receptor (Rajagopalan et al., 1996). The angiotensin AT_1 receptor also mediates synthesis of pro-inflammatory cytokines and adhesion molecules (Tummala et al., 1999). Recent studies suggest benefits of angiotensin AT_1

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antagonists beyond a reduction in blood pressure (Lindholm et al., 2002). In this regard, angiotensin AT_1 antagonists could diminish the production of superoxide anions, leading to protective effects in diabetics. However, in recent clinical trials, various β-adrenoceptor antagonists have been proven to be also of prognostic benefit in diabetic patients (Haas et al., 2003). This finding gives rise to the hypotheses regarding the mechanisms underlying the beneficial effect of β-adrenoceptor antagonists in diabetic subjects. This study was performed to compare blood-pressure-independent effects of the angiotensin AT_1 receptor antagonist candesartan and the selective β_1-adrenoceptor antagonist metoprolol on oxidative stress in streptozotocin-induced diabetic rats. To elucidate pathways mediating protective effects, the expression of several genes and proteins involved in generation of oxidative stress and inflammatory cascades were investigated in the quadriceps musculature and as a functional aspect the inherent vascular bed was characterized in vivo in the autoperfused hindlimb model.

2. Material and methods

2.1. Experimental animals

Experiments were performed in 8-week-old male Sprague–Dawley rats weighing 300–320 g (Charles River, Berlin, Germany). Rats were housed under standard conditions (20 °C, 12-h light/dark cycle) and given free access to water and standard food. The investigation conforms with 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). Diabetes mellitus was induced by a single intraperitoneal injection of 70 mg/kg of streptozotocin (Sigma, St. Louis, USA) dissolved in 0.1 mol/l citric acid–trisodium citrate buffer with a pH of 4.5 as previously described (Tschöpe et al., 1999). Control rats ($n=7$) received citrate buffer alone. After 7, 14, 21 and 48 days, blood glucose concentrations were measured using a glucometer (Accutrend sensor, Boehringer Mannheim, Germany). All animals were weighted weekly. Non-diabetic Sprague–Dawley rats served as controls (Group 1) ($n=7$). Streptozotocin-diabetic animals were further randomised to receive vehicle (10% β-cyclodextrane solution) (Group 2), the angiotensin AT_1 receptor antagonist candesartan (Astra Zeneca, Wedel, Germany) (1.5 mg/kg body weight per day by gavage) (Group 3) and the β_1-adrenoceptor antagonist metoprolol (Astra Zeneca, Wedel, Germany) (30 mg/kg body weight per day by gavage) (Group 4), using sub-antihypertensive doses, respectively ($n=8$ each group). Treatment was started 5 days after induction of diabetes and was continued for 48 days.

2.2. Hindlimb perfusion and blood pressure measurement

For the evaluation of vascular function, the autoperfused hindlimb model was taken as described before (Angulo et al., 1998). Briefly, on treatment day 48 the rats were anaesthetized with chloral hydrate (Sigma, St. Louis, USA) (400 mg/kg, i.p.). After intubation, the animals were mechanically ventilated with a respirator (Ugo Basile, Comerio, Italy) and their temperature was maintained at 37 °C with a thermic table. Under aseptic conditions, catheters (PP20) were placed in both carotid arteries. Systemic blood pressure and heart rate were recorded from the right carotid artery through a tip-catheter (2F) system (Millar, Föhr Medical Instruments, Seeheim, Germany). A femoral artery catheter was placed through a separate incision into the right groin. Perfusion of the left hindlimb was performed by means of a roller peristaltic pump (Minipuls 3, Abimed, Langenfeld, Germany), which delivered blood from the left carotid artery. Flow was set at 2.2 ml/min per kilogram bodyweight. The hindlimb perfusion pressure was recorded with a pressure transducer (Medex Inc., Carlsbad, USA) located distal to the peristaltic pump. Extracorporal volume was 1.1 ml, filled with Krebs–Henseleit solution containing sodium heparin (60 IE/ml). The flow rate was kept constant so that basal vascular resistance to flow could be compared between experimental groups. An infusion pump was connected on the cannula side of the peristaltic pump. This allowed us to obtain depressor responses to infusion of vasoactive drugs and to flow-mediated vasodilatation by infusion of Krebs–Henseleit solution.

2.3. Endothelium-dependent and -independent vasodilatation

Infusion of Krebs–Henseleit solution was used to estimate flow-mediated endothelium-dependent vasorelaxation. In order to induce graduated flow-dependent vasodilatation, three different volumes of Krebs–Henseleit solution (80, 200 and 600 μl/kg) were administered. The response elicited by each dose of Krebs–Henseleit solution was obtained from the baseline vasopressor tone, the effect of any previous infusion having entirely disappeared. When circulatory parameters were again stabilized, sodium nitroprusside (Sigma, St. Louis, USA) (40 μg/kg) was used to estimate vascular responsiveness to exogenous NO (Angulo et al., 1998). The rats were anaesthetized throughout the entire measurements.

2.4. Real-time reverse transcription-polymerase chain reaction

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Light Cycler system (Roche, Mannheim, Germany). Total RNA was isolated from each quadriceps muscle and cDNA was

Table 1
Basic metabolic and hemodynamic characterization on treatment day 48

	SD-Co (n=7)	SD-STZ (n=8)	STZ-Can (n=8)	STZ-Met (n=8)
Body weight (g)	495±7.4	250±2.5*	251±1.9*	252±2.2*
Heart weight (g)	1.63±0.09	0.85±0.05*	0.78±0.05*	0.73±0.04*
Systolic BP (mm Hg)	107±6	101±8	99±6	95±3
Diastolic BP (mm Hg)	79±7	75±5	71±7	73±2
Heart rate (beats/min)	442±29	422±25	413±31	414±23

Blood pressure values and heart rates were recorded in anaesthetized rats. Data are expressed as mean±S.E.M. Non-diabetic Sprague–Dawley (SD) rats who served as controls (Co) are indicated by SD-Co. SD-STZ stands for streptozotocin (STZ)-induced diabetic rats with vehicle-treatment. STZ-Can indicates diabetic rats treated with candesartan (Can) and STZ-Met treatment with metoprolol (Met). Number of animals used is in parentheses. BP indicates blood pressure. *P<0.05 vs. SD-Co.

generated. The following primers were used for the PCR analysis: eNOS: 5′-GTGTTTGGCCGAGTCCTCACC-3′ and 5′-CTCCTGCAAGGAAAAGCTCTG-3′, NAD(P)H oxidase $p22^{phox}$ subunit: 5′-ATGGGCAACTTGAAGAGCGTGGC-3′ and 5′-TCCATGTG GAGCCCTTCTT-3′, NAD(P)H oxidase $gp91^{phox}$ subunit: 5′-GAGGTGGGACAATA-CATTTTC-3′ and 5′-CTGCTTATCACAGCCACAGGC-3′. Gene expression was normalized in relation to the expression of an endogenous control (“house-keeping gene”), 18S ribosomal RNA (rRNA) (primer: 5′-TAGAGGGACAAGTGG-CGTTC-3′ and 5′-TGTACAAAGGGCAGGGACTT-3′).

2.5. NAD(P)H oxidase activity

Muscle NAD(P)H oxidase activity was measured by superoxide dismutase-inhibitable reduction of cytochrome *C* with NADH or NAD(P)H as substrates. The tissue homogenates were incubated at 37 °C and the absorbance at 550 nm was measured. NADH-stimulated production of reactive oxygen species was determined by following increases in cytochrome *C* absorbance. There was no measurable activity in the absence of NADH. The experiments were performed in parallel with and without superoxide dismutase. The activity of NAD(P)H oxidase was calculated as superoxide dismutase-inhibitable cytochrome *C* reduction.

2.6. Immunohistochemistry and image analysis

Tissue samples of quadriceps muscles were embedded in O.C.T. (optimal cutting temperature) compound (Tissue Tek, Sakura Finetek, USA), frozen in liquid nitrogen and stored at −80 °C. Serial 5-μm-thick cryosections were placed on 10% poly-L-lysine precoated slides and fixed in cold acetone. After blocking endogenous peroxidase activity, sections were incubated with an avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, USA). Staining was performed with the following primary antibodies at the given dilutions (45 min, room temperature): mouse-anti-rat ICAM-1 (Serotec, Oxford, UK; 1:100), mouse-anti-rat VCAM-1 (HISS Diagnostics, Freiburg, Germany; 1:50), rabbit-anti-rat iNOS and rabbit-anti-rat eNOS (both Alpha Diagnostic, San Antonio, USA; 1:50). The first two antibodies were detected using a biotinylated goat-anti-mouse IgG (Dianova, Hamburg, Germany; 1:400), the latter two by biotinylated goat-anti-rabbit IgG (Dianova, Hamburg, Germany; 1:333). Sections were incubated with peroxidase-conjugated streptavidin (Vectastatin ABC Elite, Vector Laboratories Inc, Burlingame, USA) (30 min, room temperature). Antigen–antibody complexes were visualized by 3-amino-9-ethylcarbazole (Merck, Darmstadt, Germany). The slides were counterstained (Mayer's hematoxylin), mounted in Kaiser's gelatine (both Merck, Darmstadt, Germany) and evaluated in a blinded fashion using the digital image analysis system Lucia G Version 3.52b (Nikon Deutschland GmbH, Düsseldorf, Germany) as described before (Noutsias et al., 2002).

2.7. Statistical analysis

Statistical analysis was performed using JMP Statistical Discovery Software Version 4.02 (SAS Institute, Cary, USA). All data are expressed as means±S.E.M. Statistical differences were analyzed by analysis of variants (ANOVA) in conjunction with a two-tailed Student's *t*-test. The significant difference for multiple comparisons of the immunohistochemical statistics was calculated by the

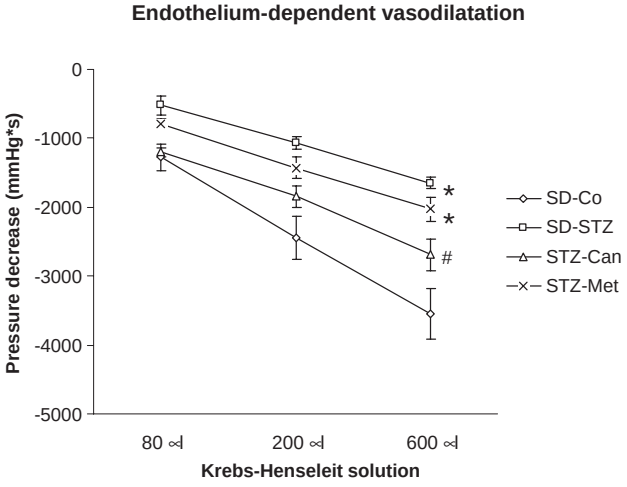

Fig. 1. Flow-mediated endothelium-dependent vasodilatation elicited by administration of Krebs–Henseleit solution. Data are expressed as mean±S.E.M and displayed as the integral of the decrease of the previous perfusion pressure of the left hindlimb circulation [mm Hg/s]. Non-diabetic Sprague–Dawley (SD) rats who served as controls (Co) are indicated by SD-Co. SD-STZ stands for streptozotocin (STZ)-induced diabetic rats with vehicle-treatment. STZ-Can indicates diabetic rats treated with candesartan (Can) and STZ-Met treatment with metoprolol (Met). *P<0.05 vs. SD-Co. $^{\#}P$<0.05 vs. SD-STZ.

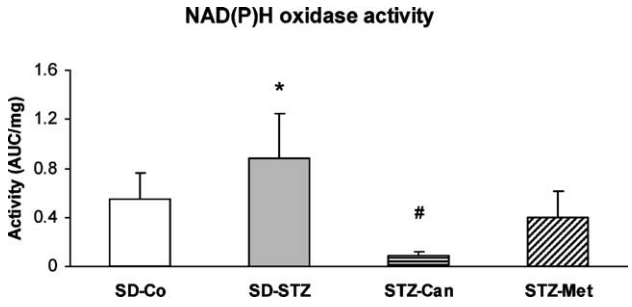

Fig. 2. Oxidative stress in control, diabetic and treated diabetic rats. Oxidative stress in terms of production of reactive oxygen species was measured in samples of rat skeletal muscle (M. quadriceps) by superoxide dismutase-inhibitable reduction of cytochrome C with NADH or NAD(P)H as substrates. Non-diabetic Sprague–Dawley (SD) rats who served as controls (Co) are indicated by SD-Co. SD-STZ stands for streptozotocin (STZ)-induced diabetic rats with vehicle-treatment. STZ-Can indicates diabetic rats treated with candesartan (Can) and STZ-Met treatment with metoprolol (Met). *$P<0.05$ vs. SD-Co. $^{\#}P<0.05$ vs. SD-STZ.

Tukey–Kramer analysis. Differences were considered statistically significant at a value of $P<0.05$.

3. Results

3.1. Basic parameters

Blood glucose levels, body and heart weights were measured in all of the animals studied. After streptozotocin administration blood glucose increased regularly to a level greater than 27.7 mmol/l (data not shown). Diabetic animals gained less weight than non-diabetic control animals (Table 1). None of those parameters were altered by candesartan or metoprolol (Table 1). Systemic arterial pressures and heart rates were similar in both non-diabetic and diabetic vehicle-treated rats and were not affected by candesartan or low-dose metoprolol treatment.

3.2. Endothelium-dependent vasodilatation

Infusion of Krebs–Henseleit solution led to a flow-mediated endothelium-dependent vasodilatation in the vascular bed of the hindlimb in every group of rats studied. The grade of vasodilatation correlated with the volume applied and produced a transitory fall of the perfusion pressure in the hindlimb circulation. Maximal vasorelaxations were achieved by applying 600 µl/kg of the solution. As shown in Fig. 1, vasodilatation was significantly reduced in the rats with streptozotocin-induced diabetes as compared to non-diabetic controls. At volumes of 80, 200 and 600 µl/kg, the integral of perfusion pressure only decreased by 518±137 (−59.5%), 1069±90 (−56.4%) and 1650±84 mm Hg/s (−53.5%) ($P<0.05$ vs. non-diabetic control rats). As one of the main findings of our study, flow-mediated vasodilator response was significantly increased in candesartan-treated diabetic rats compared to vehicle-treated diabetic rats. Fig. 1 shows that metoprolol treatment had no effect on endothelium-dependent vasodilatation compared to vehicle-treated diabetic rats.

3.3. Endothelium-independent vasodilatation

The NO-mediated endothelium-independent vasodilatation was assessed using sodium nitroprusside to stimulate the vascular smooth muscle cells directly. Vasodilatation caused by sodium nitroprusside, expressed as integral of perfusion pressure decrease, was significantly impaired in diabetic rats with 3554±755 vs. 6602±953 mm Hg/s for non-diabetic control rats. Whether candesartan or metoprolol treatment had any effect on sodium nitroprusside-mediated vasomotion under diabetic conditions (3506.3±499.9 mm Hg/s for candesartan, 3373.3±524.3 mm Hg/s for metoprolol; not significant compared to diabetic control rats).

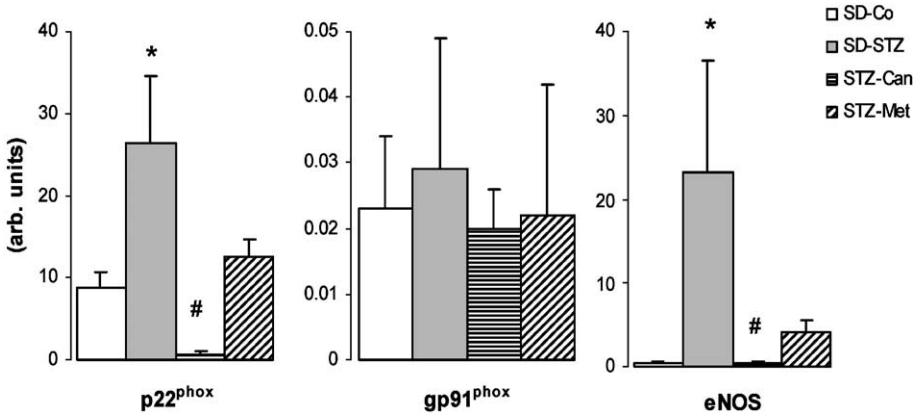

Fig. 3. mRNA expression of NAD(P)H oxidase subunits ($p22^{phox}$ and $gp91^{phox}$) and of endothelial nitric oxide synthase (eNOS). Transcripts of NAD(P)H oxidase subunits and of eNOS were evaluated by real-time polymerase chain reaction. Non-diabetic Sprague–Dawley (SD) rats who served as controls (Co) are indicated by SD-Co. SD-STZ stands for streptozotocin (STZ)-induced diabetic rats with vehicle-treatment. STZ-Can indicates diabetic rats treated with candesartan (Can) and STZ-Met treatment with metoprolol (Met). *$P<0.05$ vs. SD-Co. $^{\#}P<0.05$ vs. SD-STZ.

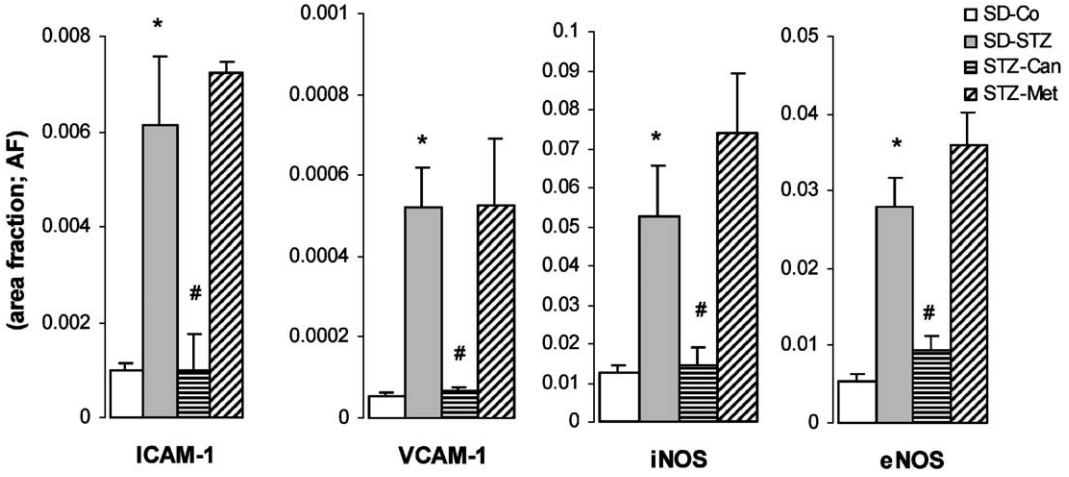

Fig. 4. Protein expression of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, inducible nitric oxide (NO) synthase (iNOS) and endothelial NO synthase (eNOS), measured by semi-quantitative immunohistochemistry and expressed as area fraction (AF) [%]. Non-diabetic Sprague–Dawley (SD) rats who served as controls (Co) are indicated by SD-Co. SD-STZ stands for streptozotocin (STZ)-induced diabetic rats with vehicle-treatment. STZ-Can indicates diabetic rats treated with candesartan (Can) and STZ-Met treatment with metoprolol (Met). *$P<0.05$ vs. SD-Co. $^{\#}P<0.05$ vs. SD-STZ.

3.4. NAD(P)H oxidase activity

Oxidative stress in terms of production of reactive oxygen species was measured by superoxide dismutase-inhibitable reduction of cytochrome *C* with NADH or NAD(P)H as substrates 48 days after induction of diabetes mellitus. Quadriceps muscle tissue of diabetic rats showed significant increases in NAD(P)H activity resulting in higher levels of reactive oxygen species (0.88±0.37 vs. 0.55±0.21 [AUC/mg]; $P<0.05$). Candesartan but not metoprolol was associated with a marked reduction in NAD(P)H oxidase activity in diabetic rats (Fig. 2).

3.5. Gene expression of $p22^{phox}$, $gp91^{phox}$ and eNOS

To further elucidate the role of NAD(P)H oxidase we examined two subunits ($p22^{phox}$ and $gp91^{phox}$) of the multienzyme complex. As shown in Fig. 3, $p22^{phox}$ mRNA was upregulated in vehicle-treated diabetic rats (8.8±1.9 vs. 26.3±8.2 [arb. units]; $P<0.05$), whereas the changes in $gp91^{phox}$ expression were not significant 48 days after induction of the diabetic state (0.023±0.011 vs. 0.029±0.02; n.s.). Treatment with candesartan but not metoprolol led to decreased $p22^{phox}$ mRNA levels in diabetic rats. Expression of $gp91^{phox}$ mRNA remained unchanged during treatment with candesartan and metoprolol. RT-PCR and immunohistochemistry were performed to evaluate the inducibility of eNOS expression in skeletal muscle by diabetic conditions (immunohistological results see below). Compared with non-diabetic controls, the level of eNOS-mRNA was significantly increased in vehicle-treated diabetic rats (0.43± 0.18 vs. 23.1±13.4; $P<0.05$) (Fig. 3). Candesartan (1.5 mg/kg) but not metoprolol led to a significantly decreased eNOS-mRNA expression level.

3.6. Immunoreactivity of ICAM-1, VCAM-1, eNOS and iNOS

To examine the downstream effects of enhanced oxidative stress in diabetes mellitus, expression of several pro-inflammatory mediators was measured. Diabetic rats demonstrated a marked increase (6-fold) in ICAM-1 protein expression compared to non-diabetic-controls (0.00338±0.00137 vs. 0.00049±0.00013 [% area fraction], $P<0.05$, Fig. 4). Diabetic rats treated with candesartan exhibited a significant lower ICAM-1 immunoreactivity, whereas metoprolol had no effect on ICAM-1 expression. As shown in Fig. 4, VCAM-1 expression was also markedly increased under diabetic conditions (10-fold, 0.00288±0.00101 vs. 0.00026±0.00007, $P<0.05$) and revealed prominent immunoreactivity localized on the endothelium (Fig. 5). Candesartan treatment significantly reduced VCAM-1 protein expression. Metoprolol showed no effect on VCAM-1 expression. Diabetic rats had a significantly higher iNOS-expression (0.05244±0.02323 vs. 0.0127±0.00179; $P<0.05$, Fig. 4) and eNOS-expression (0.02805±0.00371 vs. 0.00549±0.00095; $P<0.05$, Fig. 4) compared with non-diabetic control rats. Treatment with candesartan but not metoprolol led to a normalisation of iNOS and eNOS protein expression levels in quadriceps muscle tissue of diabetic rats (Fig. 4).

4. Discussion

The present study clearly demonstrates that the angiotensin AT_1 receptor antagonist candesartan, in contrast to the β_1-selective adrenoceptor antagonist metoprolol, unfolded protective effects in experimental diabetes mellitus, even at a low dose that did not affect blood pressure. The diabetic state

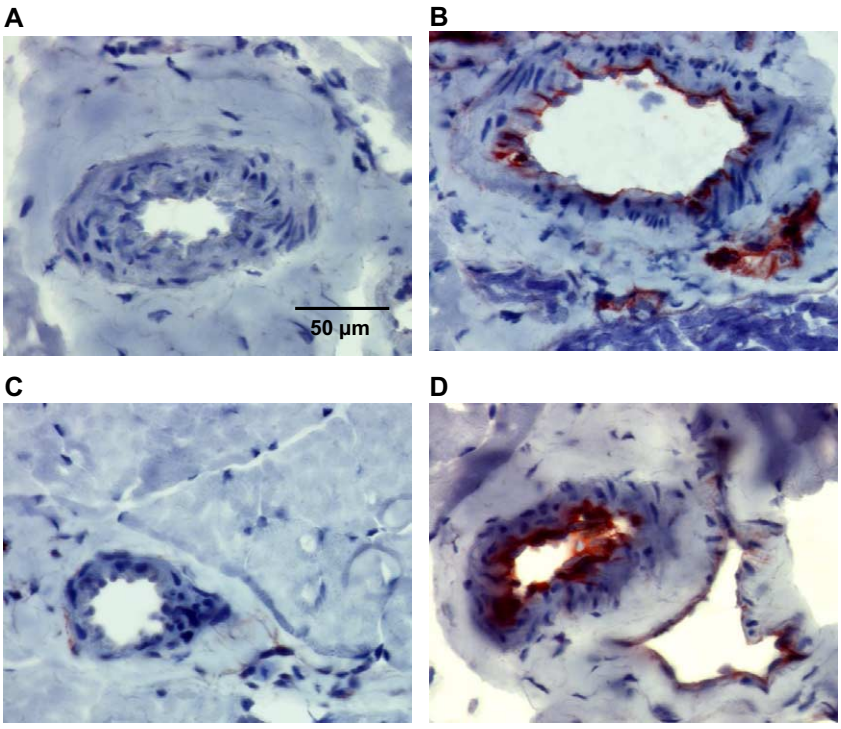

Fig. 5. Representative aspects of VCAM-1 expression in rat quadriceps muscle. Non-diabetic control rats (A), diabetic rats treated with vehicle (B), candesartan (C) and metoprolol (D). VCAM-1 immunoreactivity is clearly present in the endothelium (magnification, ×630).

led to an enhanced level of oxidative stress accompanied by an increased eNOS-mRNA expression, increased ICAM-1, VCAM-1, iNOS and eNOS protein expression and impaired endothelium-dependent vasodilatation which was antagonized by candesartan, but not by metoprolol.

We examined levels of oxidative stress and inflammation in the quadriceps muscle of streptozotocin-induced diabetic rats. It is widely accepted that the diabetic state is combined with enhanced oxidative stress in various tissues, e.g. myocardial and skeletal muscle tissue (Hsueh and Quinones, 2003). In the present study we found increased NAD(P)H oxidase activity and increased expression of the NAD(P)H oxidase subunit $p22^{phox}$ which is in agreement with previous works (Zhang et al., 2003). It has been shown that NAD(P)H generates a great amount of superoxide anions and that the superoxide production in the streptozotocin-model is directly related to the level of hyperglycemia (Hink et al., 2001; Pieper et al., 1995). Based on the current data, we were also able to demonstrate that increased activity and expression of NAD(P)H were paralleled by an increased expression of eNOS. Both at mRNA and protein level, the enzyme was markedly upregulated in diabetic rats compared to non-diabetic controls. Identical findings of increased eNOS expression have been reported before in diabetic rats (Hink et al., 2001) and were confirmed in a more recent report (Bitar et al., 2005). Extensive work on the cellular and molecular level has been performed previously to understand this apparent paradox of diabetes-induced super induction of eNOS concomitantly with endothelial dysfunction. It has become clear that in the setting of diabetes, upregulated eNOS is dysfunctional or "uncoupled" and may be due to a lack of the substrate L-arginine or the cofactor tetrahydrobiopterin (Hsueh and Quinones, 2003; Mollnau et al., 2002; Bojunga et al., 2004; Thomsen et al., 2002). In the uncoupled state, electrons flowing from the reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine, resulting in production of oxygen radicals rather than NO (Vasquez-Vivar et al., 1998; Xia et al., 1998). In the light of these previous analyses, enzyme uncoupling is a reasonable explanation for our observation of an upregulated eNOS in combination with endothelial dysfunction. Enhanced superoxide production is known to lead secondarily to degradation of NO. Both NO and superoxide anions are unstable radicals, which react very rapidly to form the main product peroxynitrite, which in turn is the main mediator of oxidative damage (Coppey et al., 2001). In the present study, we also found an increased iNOS expression. NO derived from iNOS can function as a pro-inflammatory mediator and by reacting with superoxide anions it has the potential to aggravate oxidative stress. It is well established that iNOS synthesizes 10- to 50-fold more NO than the constitutive eNOS and that its expression is increased in the setting of diabetes (Bardell and MacLeod, 2001). The altered redox state is further accompanied by the upregulation of inflammatory cytokines, chemokines and adhesion molecules. Glycosylated proteins, among them advanced glycation end products, can augment the production of pro-inflammatory cytokines and of leukocyte adhesion

molecules (ICAM-1, VCAM-1) as well as other inflammatory pathways in vascular endothelial cells (Schmidt et al., 1999). As pointed out above, we were able to demonstrate enhanced vascular protein expression of ICAM-1 and VCAM-1 under diabetic conditions. Furthermore, the relaxant responses evoked by sodium nitroprusside, an endothelium-independent exogenous NO donor, were also diminished under diabetic conditions. This finding of our experiments is in agreement with other studies demonstrating an impaired response to exogenous NO in the diabetic state in animals and humans (Angulo et al., 1998; O'Driscoll et al., 1999).

Low-dose candesartan treatment led to a significant improvement of flow-mediated endothelium-dependent vasodilator response in diabetic rats. This finding is consistent with previous studies showing endothelial protective effects by angiotensin AT_1 receptor antagonism in patients with hypercholesterolemia, hypertension and coronary artery disease (Wassmann et al., 2002; Schiffrin et al., 2000; Hornig et al., 2003). Moreover, our results show that candesartan did not influence endothelium-independent vasodilatation, which is in agreement with previous works, demonstrating that inhibition of the renin–angiotensin system with angiotensin-converting enzyme (ACE) inhibitors and angiotensin AT_1 receptor antagonists does not influence endothelium-independent vasodilatation (O'Driscoll et al., 1999; Hornig et al., 2003). Our data further suggest a pivotal role of angiotensin II for the development of diabetes-induced endothelial dysfunction. Both local vascular angiotensin II production and angiotensin AT_1 receptor expression are significantly increased in diabetic rats (Sechi et al., 1994). Although the mechanisms of action underlying the role of the renin–angiotensin system and the angiotensin AT_1 receptor in diabetes-induced endothelial dysfunction are not completely understood, a plausible explanation is that hyperglycemia promotes an activation of the angiotensin AT_1 receptor. This activation leads to a stimulation of the NAD(P)H oxidase and consequently to increased levels of reactive oxygen species, such as superoxide anions (Mollnau et al., 2002). Our study shows that treatment with candesartan reduces oxidative stress levels in skeletal muscle tissue by reducing NAD(P)H oxidase activity to subnormal levels, down-regulation of eNOS-mRNA expression, and reducing protein expression of eNOS and iNOS. These effects of angiotensin AT_1 receptor antagonism occurred without affecting blood pressure and blood glucose levels. It has been suggested that many of the blood pressure-independent ancillary or so-called “pleiotropic” effects of angiotensin AT_1 receptor antagonists may be caused by antioxidant properties (Chung and Unger, 1999). Previous studies have reported that treatment with candesartan reduces oxidative stress in patients with essential hypertension independent of its effects on blood pressure (Dohi et al., 2003). This finding is consistent with those of others, showing that candesartan reduces NAD(P)H oxidase activity in streptozotocin-diabetic rats (Kusaka et al., 2004). Our results are also in agreement with recent data demonstrating that candesartan attenuates the expression of NAD(P)H oxidase subunits and suppresses the formation of endothelial superoxide anions (Rueckschloss et al., 2002). Interestingly, other groups also found only an upregulation of NAD(P)H oxidase $p22^{phox}$, while changes in $gp91^{phox}$ remained insignificant (Zhang et al., 2003). In summary, the available data suggest a strong antioxidative potential of candesartan by downregulation of NAD(P)H oxidase expression and activity. In this study, we also examined if candesartan can protect against the downstream effects of enhanced oxidative stress, namely enhanced neutrophil-endothelium cell adhesion through increased expression of endothelial ICAM-1 and VCAM-1. Both adhesion molecules are important markers of endothelial dysfunction and are intimately linked to the onset of atherosclerotic processes. In agreement with previous studies, the present results demonstrate enhanced endothelial expression of ICAM-1 and VCAM-1 in diabetic rats (Zhang et al., 2003). We showed significant reductions in ICAM-1 and VCAM-1 protein expression following 48 days of treatment with low-dose candesartan. In this context, angiotensin AT_1 receptor antagonist-induced reductions in inflammatory markers were independent of decreases in blood pressure or blood glucose. Induction of adhesion molecules through angiotensin II via the AT_1 receptor has been described in a rat model (Tummala et al., 1999). In the same model, increased expression of VCAM-1 was antagonized by an angiotensin AT_1 receptor antagonist. Moreover, candesartan has been shown to reduce levels of circulating soluble adhesion molecules (Dohi et al., 2003). However, to our knowledge, this is the first time that an immunohistological examination of the tissue expression pattern of ICAM-1 and VCAM-1 has been performed in an in vivo haemodynamically characterized peripheral vasculature bed.

Metoprolol failed to show any beneficial effects in our study. Only few studies have been performed with a focus on the therapy or prevention of endothelial dysfunction employing beta-adrenoceptor antagonists and the available data are conflicting. Atenolol, in blood pressure-influencing doses, improved endothelial function in hypertensive subjects but failed to show any effect in another study (Schiffrin et al., 2002; Arosio et al., 2002). In streptozotocin-diabetic rats, celiprolol showed a protective effect on the development on endothelial dysfunction, which seems to be due to a β_2-adrenoceptor-mediated mechanism (Olbrich et al., 1999; Kabat et al., 2004). Carvedilol, known to exert anti-oxidative properties, has been shown to improve endothelial function in diabetic subjects, independent of blood pressure reduction (Giugliano et al., 1998). Thus, beta-adrenoceptor antagonists are a very heterogeneous class of drugs with differing modes of action (e.g. β_1-selective agents like nebivolol, bisoprolol and metoprolol or non-selective vasodilating blockers such as carvedilol). However, although NAD(P)H oxidase activity, $p22^{phox}$ and eNOS

mRNA expression seemed to be normalized after treatment with metoprolol, metoprolol could neither influence levels of inflammation under streptozotocin-induced diabetic conditions nor could improve endothelial function. In a substudy, we found that even metoprolol at a higher dose (50 mg/kg body weight per day), potent enough to reduce heart rate and blood pressure, did not also lead to an improvement in endothelial function (data not shown). Altogether this is a surprising result since we expected that an improvement of oxidative stress indices would consequently lead to an improvement of endothelial function. Several hypotheses are possible to explain this discrepancy: we determined NAD(P)H oxidase activity and $p22^{phox}$ mRNA expression in the muscle and not in isolated vascular tissue. It is possible that metoprolol differs in the regulation of oxidative stress enzymes in different tissues. However, endothelial dysfunction was not influenced by metoprolol treatment and correlated with enhanced expression of vascular adhesion molecules, which reinforces this suggestion. Further, only treatment with candesartan led to a reduction of oxidative stress indices below levels of control animals. Regarding the complex pathogenesis of endothelial dysfunction under diabetic conditions, a reduction of some oxidative stress indices to basal levels might not sufficient enough to improve vascular function. On the other hand, metoprolol did not aggravate the metabolic state or lead to an acceleration of angiopathic processes in our diabetic rats. Further studies are necessary to clarify these results. According to our data, a selective β_1-adrenoceptor antagonist is not sufficient to improve endothelial function in streptozotocin-induced severely diabetic rats.

The major limitation of the present study is that a streptozotocin-rat model of diabetes does not fully represent the entire spectrum of complex metabolic abnormalities seen in diabetes mellitus of humans. Furthermore, enzyme activity and gene and protein expression were measured in quadriceps muscle, which is known to consist of different tissue types. Regarding the involved mechanism, investigation of isolated vessels would have increased the impact of our results. As beta-adrenoceptor antagonists differ in their pharmacologic characteristics, it is also not clear whether our results represent a class effect or whether this effect is only specific to metoprolol.

In conclusion, our study demonstrates the presence of endothelial dysfunction and enhanced oxidative stress combined with an augmented expression of inflammatory markers in streptozotocin-diabetic rats. Treatment with the angiotensin AT_1 receptor antagonist candesartan in a sub-antihypertensive dose normalized endothelium-dependent vasodilatation, decreased oxidative stress and prevented the initiation of inflammatory processes. As the β_1-selective adrenoceptor antagonist metoprolol failed to evoke these beneficial effects, it appears likely that, under diabetic conditions, the renin–angiotensin system plays a crucial role in mediating the development of endothelial dysfunction, which is thought to be a pre-atherosclerotic state.

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