INTRODUCTION

Cardiovascular disease is the primary cause of morbidity and mortality among patients with diabetes and accounts for more than 80% of deaths in this patient population (1). Abnormal endothelial function, platelet hyperreactivity, aggressive atherosclerosis, and adverse arterial remodelling develop very early in patients with diabetes (1). Key determinants of endothelial dysfunction are reduced nitric oxide (NO) bioavailability and abundant formation of reactive oxygen species (ROS) within the vascular wall resulting in an imbalance between NO and ROS (2). In addition to its effects on vascular tone, NO is a central regulator of platelet activation, adhesion and aggregation. Acute as well as chronic reduction of systemic NO bioavailability precipitates platelet activation in vivo (3, 4).

Based on the elevated cardiovascular and renal risk of diabetic patients, suitable pharmacologic treatments that offer protection from diabetic complications are needed, particularly in light of the growing number of patients developing this disease. The rennin-angiotensin-aldosterone system (RAAS) essentially contributes to the regulation of systemic vascular homeostasis. RAAS inhibition by angiotensin-converting enzyme (ACE) inhibition or angiotensin II receptor blockade reduces target organ damage and cardiovascular complications in patients with diabetes (5-7). In animals treated with RAS inhibitors inflammatory changes and fibrosis in chronic pancreatitis were attenuated and apoptosis of pancreatic acinar cells was alleviated, so RAAS blockade might potentially have beneficial effects on the pancreas (8).

Diabetic nephropathy is the most common cause of end-stage renal disease. In diabetic rats, mineralocorticoid receptor (MR) blockade by spironolactone ameliorates renal injury (9) and prevents diabetic nephropathy by anti-inflammatory mechanisms (10). The selective MR blocker eplerenone reduced renal injury in diabetic rodent models (11). In diabetic patients, spironolactone as well as eplerenone successfully diminished albuminuria (12, 13) and reduced the urinary excretion of markers for oxidative stress (14). In addition to its detrimental effects on the kidney, aldosterone directly activates NADPH oxidase and inhibits eNOS phosphorylation/activation (15, 16) in vascular cells, which disturbs the balance of NO and ROS leading to endothelial dysfunction and atherogenesis (15, 16). Under conditions of increased RAAS activation such as heart failure, MR blockade reduces oxidative stress, improves endothelial function, and increases systemic NO bioavailability (17, 18). However, it is unclear whether MR blockade beneficially affects vascular dysfunction in diabetes.

Therefore, in the present study, we examined the effect of eplerenone on vascular function, superoxide formation, NO bioavailability, and platelet activation in diabetic rats.
METHODS

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) and current guidelines at the University of Würzburg, Germany.

Animals and induction of diabetes

Male Wistar rats (250-300 g, obtained from Harlan-Winkelmann, Borchen, Germany) were housed in temperature-controlled cages (20-22°C) with a 12-hour light-dark cycle, and given free access to water and formulated diets. A single dose streptozotocin regimen was used to induce pancreatic-islet-cell destruction and persistent hyperglycaemia. Streptozotocin (10 mg/mL, Sigma, Deisenhofen, Germany) was freshly dissolved in sterile sodium citrate buffer (25 mmol/L, pH 4.5) and used within 10 minutes. Rats received a single 50 mg/kg intravenous injection of streptozotocin or citrate buffer (control). Blood glucose was monitored using a one-touch blood glucose meter (Ascensia Elite, Bayer-Vital GmbH, Leverkusen, Germany). Hyperglycaemia was defined as a random blood glucose level >20 mmol/L at 2 and 4 weeks after injection. Rats were randomized to placebo or eplerenone (100 mg/kg per day by gavage, Pfizer) at day 14. Two weeks later, vasomotor function and platelet activation were assessed.

Platelet sampling and flow cytometry

Deep general anesthesia was induced using isoflurane, determined by total absence of reaction to pain under spontaneous respiration. The abdominal cavity was opened and blood was taken by direct puncture of the inferior caval vein into a tube containing 3.8% sodium citrate. Whole blood was diluted with PBS and stained with fluorescein-isothiocyanate-labelled antibodies to detect platelet surface-expression of P-selectin and platelet-bound fibrinogen (18, 19).

Afterwards, samples were analyzed on a BectonDickinson FACS Calibur. In principle, platelets were identified based on their forward (FSC)- and sideward (SSC)-scatter as depicted in a representative dot plot (Fig. 1A). All subsequent measurements (histograms) were performed in the gated area (R1) only. After adjusting the mean fluorescence with an irrelevant antibody to an arbitrary value of 10, the samples with the specific antibodies were processed, as exemplified for platelet-bound fibrinogen (Fig. 1B). Hereby, the mean fluorescence intensity (MFI) was calculated as demonstrated (M1). To verify that R1 contained a high purity of platelets, separate samples were labelled with an antibody against a platelet-specific antigen such as CD42 (Fig. 1C). The percentage of CD42⁺ cells in R1 was determined by dividing M2 by M1.

Vascular reactivity studies

After exsanguination, the descending thoracic aorta was dissected following removal of the heart and cleaned of connective tissue. Rings were cut into 3 mm rings, which were mounted in an organ bath (Foehr Medical Instruments, Seeheim, Germany) for isometric force measurements as previously described (17, 20). The rings were equilibrated for 30 min under a resting tension of 2 g in oxygenated (95% O₂; 5% CO₂) Krebs-Henseleit solution (NaCl 118 mmol/L, KCl 4.7 mmol/L, MgSO₄ 1.2 mmol/L, CaCl₂ 1.6 mmol/L, KH₂PO₄ 1.2 mmol/L, NaHCO₃ 25 mmol/L, glucose 12 mmol/L; pH 7.4, 37°C) containing

Fig. 1. Identification of platelets based on their forward (FSC)- and sideward (SSC)-scatter (A). Subsequent measurements (histograms) were performed in the gated area (R1) only. The mean fluorescence intensity (MFI) was calculated as demonstrated in samples with the specific antibodies (B). To verify that R1 contained a high purity of platelets, separate samples were labelled with an antibody against the platelet-specific antigen CD42 (C). The percentage of CD42⁺ cells in R1 was determined by dividing M2 by M1.
diclofenac (1 µmol/L). Rings were repeatedly contracted by KCl (with a maximum of 100 mmol/L) until reproducible responses were obtained.

The relaxant response to cumulative concentrations of acetylcholine was assessed after preconstriction with phenylephrine to comparable levels. Afterwards, aortic rings were slightly preconstricted to about 20% of the maximal constriction with low, incremental concentrations of phenylephrine and the additional contraction to $N^G$-nitro-L-arginine was measured as a marker of physiological stretch-induced, calcium-independent NO formation (21). Furthermore, relaxant responses to the endothelium-independent vasodilator 2-(N,N-Diethylamino)-diazenolate-2-oxide (Alexis Biochemicals, San Diego, CA) were determined after preconstriction with phenylephrine in the presence of $N^G$-nitro-L-arginine.

Measurement of superoxide anion formation

One aortic segment was used for measurement of superoxide production by lucigenin-enhanced chemiluminescence (17). The light reaction between superoxide and lucigenin (5 µmol/L) was detected in a luminometer (Wallac, Freiburg, Germany) during incubation of rings in a HEPES-modified Krebs buffer (pH 7.40). To achieve more specific determination of superoxide formation, aortic segments were incubated with dihydroethidium, and 2-hydroxyethidium formation was measured using high-performance liquid chromatography as recently described (22, 23).

Substances

Unless otherwise stated, all chemicals were obtained from Sigma (Deisenhofen, Germany) in the highest purity available.

Statistics

Values are means±standard error of means for curves and bar graphs. Relaxant responses were given as percentage relaxation relative to the preconstriction level. Statistical analysis was performed by repeated measures analysis of variance followed by Tukey-Kramer multiple comparisons test. Superoxide formation and platelet activation were analyzed by analysis of variance followed by a Tukey post-hoc test where appropriate; P<0.05 was considered statistically significant.

RESULTS

Blood glucose levels and body weight as well as platelet and leukocyte counts are shown in Table 1. Streptozotocin-induced increases in blood glucose and reduction in body weight were unaffected by MR antagonism. Platelet and leukocyte numbers were similar in all groups.

Vascular reactive oxygen species

Aortic superoxide production, assessed by lucigenin-enhanced chemiluminescence, was significantly increased in rats with diabetes and reduced by treatment with eplerenone (Fig. 2A). A separate aortic ring from each animal was measured in the presence of $N^G$-nitro-L-arginine to determine whether superoxide generation might be potentially NOS-derived (Fig. 2A).

Superoxide generation in vascular rings was detected more specifically by 2-hydroxyethidium formation using high-performance liquid chromatography and was significantly increased in aortae from diabetic vs. control animals and markedly reduced in diabetic rats treated with eplerenone (Fig. 2B).

Vasomotor function

Administration of acetylcholine in cumulative concentrations for calcium-dependent activation of endothelial NO synthase induced an endothelium-dependent vasorelaxation, which was impaired in diabetes and significantly improved by selective MR antagonism with eplerenone (Fig. 3A and 3C).

The concentration response curve for the NO donor 2-(N,N-Diethylamino)-diazenolate-2-oxide, which was used to assess endothelium-independent vasorelaxation, was shifted to the right in aortae from diabetic rats. Endothelium-independent relaxation

<table>
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<th>Control placebo</th>
<th>STZ placebo</th>
<th>STZ EPL</th>
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<tr>
<td>Blood glucose [mmol/L]</td>
<td>7.8±0.4</td>
<td>27.8±0.6*</td>
<td>26.4±1.6*</td>
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<td>Body weight [g]</td>
<td>354±5.6</td>
<td>243±3.6*</td>
<td>244±9.4*</td>
</tr>
<tr>
<td>Platelets [x1000/µl]</td>
<td>660±40</td>
<td>545±47</td>
<td>582±63</td>
</tr>
<tr>
<td>Leukocytes [x1000/µl]</td>
<td>5.5±0.4</td>
<td>4.9±0.5</td>
<td>5.5±0.6</td>
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* = p<0.01 vs. control placebo.

Fig. 2. Superoxide production in aortic rings from control rats and diabetic (STZ) rats treated either with placebo or eplerenone (EPL), * = P<0.01 vs. control placebo.
Fig. 3. Concentration-response curves for endothelium-dependent, NO-mediated vasorelaxation elicited by cumulative application of acetylcholine (A) and endothelium-independent relaxation by incremental concentrations of 2-(N,N-Diethylamino)-diazenolate-2-oxide (DEA-NONOate, B) in isolated aortic rings from control rats (○) and diabetic rats (STZ) treated either with placebo (●) or eplerenone (EPL, ■). Respective EC<sub>50</sub> values were determined for every single concentration response of acetylcholine (C) and 2-(N,N-Diethylamino)-diazenolate-2-oxide (D). Data are means±SEM from 10-16 different animals, ** = P<0.01 vs. control; ## = P<0.01 vs. STZ-placebo.

Fig. 4. Additional increments in vasomotor tone in slightly preconstricted aortic rings (approximately 20% of maximal constriction) from control rats and diabetic rats (STZ) treated either with placebo or eplerenone (EPL) following NO synthase-inhibition with N<sup>ω</sup>-nitro-L-arginine (L-NNA) were used as an index of vascular contraction-induced calcium-independent NO formation (A). Representative tracings are shown for each treatment group (B). Results are expressed as the mean fluorescence SEM from 10-16 separate animals, ** = P<0.01 vs. control, ## = P<0.01 vs. STZ-placebo.
was significantly improved (leftward shift) following treatment with eplerenone (Fig. 3B and 3D).

**Vascular NO bioavailability**

We assessed calcium-independent NO release in diabetic aortae by inhibition of tonic NO release using N\textsubscript{G}-nitro-L-arginine in slightly preconstricted aortic rings as previously described as a marker for NO release by physical stimuli (21). This caused an additional contraction, which was attenuated in animals with diabetes. In eplerenone-treated diabetic animals, N\textsubscript{G}-nitro-L-arginine-induced constriction was increased to levels comparable with control rats (Fig. 4A) as shown in the representative traces from each group (Fig. 4B).

**Platelet activation**

We previously demonstrated a causal relationship between endothelial dysfunction and platelet activation in diabetes (4, 19). The extent of in vivo platelet-binding of fibrinogen as a marker for activation of glycoprotein IIb/IIIa (Fig. 5A, C) and surface-expression of P-selectin (CD62P, B) on platelets from control rats or diabetic rats (STZ) treated either with placebo or eplerenone (EPL). Results are expressed as the mean fluorescence SEM from 10-16 separate animals. * = \( P<0.05 \) vs. control; * = \( P<0.05 \) vs. STZ-placebo. Typical flow cytometry histograms for platelet-bound fibrinogen (C) and P-selectin (D) show the rightward shift in diabetes (red curve) and a leftward shift following treatment with eplerenone (blue line) compared to control (filled grey).

**DISCUSSION**

In this study, we demonstrate that the selective MR blocker eplerenone improves vascular function and reduces platelet activation in experimental diabetes. The findings were attributable to reduced superoxide formation and improved NO bioavailability.

Patients with diabetes have a marked increase in the risk of coronary heart disease contributing to higher cardiovascular mortality (24). The overall number of diabetic patients with advanced renal disease is rapidly increasing. The presence of albuminuria predicts overt nephropathy and cardiovascular disease, and indicates poor prognosis (25).

Antihypertensive treatment, especially by RAAS blockade, reduces albuminuria (25). In the HOPE trial, ACE inhibition significantly lowered the risk for cardiovascular events as well as overt nephropathy in people with diabetes representing a vasculoprotective and renoprotective treatment (26). While it was originally assumed that ACE inhibitors block angiotensin II-dependent adrenal aldosterone secretion, a so-called "aldosterone escape" occurs after prolonged ACE inhibitor therapy (27). Such "aldosterone escape" with increased plasma levels of aldosterone is an independent risk factor for worse outcome in patients with heart failure (28). Similarly, aldosterone breakthrough is observed in a substantial number of diabetic hypertensive patients and is associated with a rebound of urinary albumin excretion (29).
Aldosterone itself contributes to the progression of renal disease (30) and spironolactone reduces vascular injury (31), has antifibrotic effects ameliorating renal injury (9) and prevents diabetic nephropathy in diabetic rats (10). Eplerenone similarly reduced renal injury in diabetic rodent models (11). Hyperglycaemia increases production of hyaluronan in cultured kidney cells implying that diabetes promotes induction of hyaluronan in the kidney. Papillary hyaluronan is elevated in diabetic rats coinciding with hyperglycaemia, glucosuria, proteinuria and overt diuresis (32). Both MR blockers reduced albuminuria in diabetic patients (12, 13). As full doses of ACE inhibitors and angiotensin receptor blockers attenuate but do not abrogate progression of renal dysfunction, additive aldosterone antagonism has been propagated as a rational therapeutic strategy for retarding renal disease progression based on the efficacy of MR blockade in abrogating proteinuria (33). Thus, aldosterone antagonism emerges as a potential nephro- and vasoprotective treatment option in diabetes.

In the vasculature, aldosterone promotes sympathoadrenergic activation, vessel inflammation, oxidative stress, endothelial dysfunction, and cardiovascular fibrosis and hypertrophy (34). STZ-induced diabetes leads to a marked rise in ROS activity not only in smooth muscle cells of the vasculature, but also in non-vascular smooth muscle cells such as the esophageal mucosa. These pathomechanisms involve differential glycosylation patterns as well as modification of NO bioactivity (35). MR blockade improves endothelial function in experimental models of hypertension and heart failure (17, 36).

In the present study we demonstrate enhanced vascular NO bioavailability and improved vascular function by selective MR blockade in an experimental rat model of diabetes. The rationale to use aldosterone antagonism for vasoprotection are in vitro studies demonstrating NADPH oxidase activation by aldosterone in isolated vascular smooth muscle cells directly contributing to oxidative damage (37). Aldosterone-induced vascular constriction of rat coronary arterioles can be inhibited by scavenging of ROS indicating that inactivation of NO is a central mechanism of aldosterone action (38). Enhanced expression of NADPH oxidase subunits, increased oxidative stress, and reduced NO bioavailability in vascular smooth muscle cells has been ascribed to a complex crosstalk between MR and angiotensin II receptors not yet completely understood (15, 39, 40). Aldosterone at least partially mediates angiotensin II-induced increase in superoxide formation in the vasculature (41, 42).

Endothelial dysfunction in diabetes was documented in our study by impaired vasorelaxation following stimulation with acetylcholine as well as by decreased vasconstriction following inhibition of NO formation in slightly preconstricted arteries, which more closely resembles the stimulation of eNOS by physical stimuli in vivo (21). Impaired eNOS-activity despite unaltered protein levels has been observed during states of heightened oxidative stress. Reduced co-factor availability or impaired arginine supply may lead to situations of so-called eNOS uncoupling and enhanced superoxide generation (43). The pronounced right-shift in the dose response to exogenous NO derived from 2-(N,N-diethylamino)-diazenolate-2-oxide further indicates that either smooth muscle sensitivity to NO is reduced and/or that NO is scavenged by ROS before relaxing smooth muscle cells. Oxidative stress is a major cause of reduced endothelial NO bioavailability in diabetes (44). One potentially relevant source for ROS in the vasculature could be perivascular adipose tissue, which surrounds nearly all arteries and has high endocrine and paracrine activity. Especially under diabetic conditions, this might be a relevant source for ROS (45). We found significantly higher formation of superoxide production in aortic segments from diabetic rats compared to healthy controls. While the assessment of superoxide production by lucigenin-enhanced chemiluminescence has been criticized, the measurement of 2-hydroxyethidium formation using high-performance liquid chromatography provides a sensitive and specific determination of superoxide anions (22). Selective MR blockade with eplerenone reduced superoxide anion formation and thereby improved NO bioavailability which in turn led to the normalization of vascular relaxation. Our data provide mechanistic explanations for the enhanced coronary circulatory function by eplerenone as compared to thiazide treatment in patients with diabetes already receiving ACE inhibition (46).

Reduced oxidative stress and improved endothelial function following MR blockade were also observed in experimental atherosclerosis (47) and early after myocardial infarction (48). In rats as well as in patients with severe heart failure, spironolactone improved endothelium-dependent NO formation and endothelial dysfunction (49, 50).

Reduced NO bioactivity facilitates arterial thrombosis in animal models and in individuals with endothelial dysfunction (51). Chronically impaired NO bioavailability in animal models is associated with increased platelet activation, especially in diabetes (4, 19, 52). Activated platelets have a major impact on morbidity and mortality as most diabetic patients die from atherothrombotic events (53). In addition to macrovascular events, activated platelets contribute to microvascular occlusion, embolization of platelet-platelet- or platelet-leukocyte-aggregates, and amplification of atherosclerotic and thrombogenesis in diabetic patients (1, 44). In the present study, platelet activation determined by fibrinogen-binding on activated glycoprotein IIb/IIIa as well as surface-expression of P-selection, a marker for platelet degranulation, was significantly increased in diabetic vs. non-diabetic animals. Chronic treatment with the selective MR blocker eplerenone attenuated activation of circulating platelets as already observed following MR blockade with eplerenone in heart failure (18).

PERSPECTIVES

Increased vascular oxidative stress in diabetes reduces NO bioavailability contributing to endothelial dysfunction and platelet activation, early stages in the development of atherosclerosis (44). MR activation appears to substantially contribute to the progression of vascular and renal disease in diabetes. Although spironolactone is an effective MR blocker, progestational and anti-androgenic side effects such as gynecomastia, abnormal menstrual cycles, and impotence, limit its use. In the present study, selective MR blockade with eplerenone reduced oxidative stress, improved endothelial function and attenuated platelet activation in vivo in a rodent model of diabetes. These data support the development of selective MR blockade as an additional treatment option for the prevention and treatment of athero- and thrombogenesis in patients with diabetes.

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REFERENCES


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