Increased response of renal perfusion to the antioxidant vitamin C in type 2 diabetes

Christian Delles, Markus P. Schneider, Sebastian Oehmer, Ingrid Fleischmann, Erwin F. Fleischmann and Roland E. Schmieder

Department of Medicine IV, University of Erlangen-Nürnberg, Germany

Abstract

Background. Reactive oxygen species play a major role in the development of endothelial dysfunction. It is as yet unspecified whether increased oxidative stress contributes to endothelial dysfunction of the renal vasculature in patients with type 2 diabetes.

Methods. Renal haemodynamics were studied in 20 patients with type 2 diabetes and arterial hypertension (age 62 ± 5 years) and 20 non-diabetic hypertensive patients at baseline and following infusions of the nitric oxide synthase inhibitor, N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 4.25 mg/kg); the substrate of nitric oxide synthase, L-arginine (100 mg/kg); and the antioxidant, vitamin C (3 g, co-infused with L-arginine 100 mg/kg).

Results. The response of renal plasma flow (RPF) to L-NMMA (−54 ± 62 and −45 ± 42 ml/min/1.73 m\textsuperscript{2}; \( P = \text{NS} \)) and L-arginine (+46 ± 36 and +49 ± 25 ml/min/1.73 m\textsuperscript{2}; \( P = \text{NS} \)) was not different between diabetic and non-diabetic patients. In contrast, vitamin C induced a more pronounced increase in RPF in diabetic than in non-diabetic patients when co-infused with L-arginine (+71 ± 47 and +43 ± 33 ml/min/1.73 m\textsuperscript{2}; \( P < 0.05 \)).

Conclusions. The difference in the response of renal perfusion to an antioxidant suggests increased formation of reactive oxygen species and thereby reduced nitric oxide bioavailability in the renal vasculature of patients with type 2 diabetes.

Keywords: oxidative stress; renal perfusion; type 2 diabetes

Introduction

Diabetic nephropathy critically determines morbidity and mortality in patients with diabetes mellitus. Oxidative stress is considered a central factor in the pathogenesis of cardiovascular disease including cardiovascular and renal complications of diabetes mellitus [1]. Reactive oxygen species such as the superoxide anion reduce the bioavailability of the endothelium-derived vasodilator nitric oxide by formation of peroxynitrite [1,2]. Thereby, endothelium-dependent vasodilation is impaired, as shown for instance in the forearm vasculature of patients with type 2 diabetes [3]. This impaired endothelium-dependent vasodilation can be improved to some extent by antioxidant therapy [4]. Of note, impaired endothelium-dependent vasodilation is an independent risk factor for the development and progression of cardiovascular disease [5].

In the early stage of experimental diabetes, renal cortical superoxide production is increased [6]. Superoxide anion leads to vasoconstriction of the afferent arteriole [7]. Consequently, endothelium-dependent vasodilation of afferent arterioles is blunted in experimental diabetes but can be restored by a superoxide dismutase mimetic [8]. Increased oxidative stress has been found in patients with type 2 diabetes, particularly in patients with overt diabetic nephropathy [9]. However, a possible association between endothelium-dependent vasodilation of the human renal vasculature and oxidative stress in type 2 diabetes has not yet been examined.

Endothelium-dependent vasodilation of the human renal vasculature can be assessed by systemic administration of nitric oxide synthase inhibitors, such as N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA), and the substrate of nitric oxide synthase, L-arginine [10,11]. In addition to these techniques, we administered the antioxidant vitamin C to functionally examine the contribution of oxidative stress to nitric oxide bioavailability in the renal vasculature of patients with type 2 diabetes. Since type 2 diabetes is commonly associated
with arterial hypertension, we have chosen a group of non-diabetic hypertensive patients as a control group.

**Patients and methods**

**Patients**

Patients with type 2 diabetes who were treated in our outpatient clinic or within our training programme for patients with type 2 diabetes at high cardiovascular risk were asked to take part in the present study when they fulfilled the following inclusion criteria: age between 30 and 75 years; HbA1c < 10%; blood pressure < 180/110 mmHg; normal renal function arbitrarily defined by a serum creatinine < 1.3 mg/dl and absence of albuminuria < 500 mg/day. Exclusion criteria were any other severe renal, hepatic or cardiovascular disease. The patients were permitted to stay on their usual antidiabetic and antihypertensive medication (angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, β-receptor blockers, calcium channel blockers and diuretics in 10, four, four, eight and five patients, respectively). Patients without diabetes who took part in previous studies in our institution or who were diagnosed for arterial hypertension in our out-patient clinic were asked to serve as non-diabetic control patients at similar inclusion and exclusion criteria. A dip-stick test for albuminuria had to be negative in control subjects. However, additional quantification of albuminuria was performed in 11 out of 20 control patients. Antihypertensive therapy was discontinued in control patients 4 weeks prior to the clearance studies. Active smokers were excluded from participation. All patients refrained from taking any antioxidant supplements at least 24 h prior to the clearance study and had been fasting overnight. Diabetic patients did not take their antidiabetic medication on the morning of the clearance study, whereas antihypertensive medication was taken in the usual way. All patients gave their written informed consent prior to study inclusion. The study protocol was approved by the Clinical Investigations Ethics Committee of the University of Erlangen-Nürnberg.

**Infusion protocols**

Renal haemodynamic C parameters were determined by the constant infusion input clearance technique with inulin (Inutest®, Fresenius, Linz, Austria) and sodium para-aminohippurate (Clinalfa, Basel, Switzerland) for glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively, as previously described [10]. Briefly, after bolus infusion of inulin and sodium p-aminohippurate over 15 min and a subsequent constant infusion over 105 min, a steady state between input and renal excretion of the tracer substances was reached, and the administration of experimental substances was started in addition. RPF and GFR were standardized to body surface area. Systemic haemodynamic parameters (i.e. blood pressure and heart rate) were monitored by means of an oscillometric device (Dinamap 1846 SX; Criticon, Norderstedt, Germany).

L-NMMA was administered intravenously (i.v.) as a bolus infusion (3 mg/kg over 5 min) followed by constant infusion (1.25 mg/kg over 25 min). Thus, the total dose of L-NMMA was 4.25 mg/kg. Then, L-arginine (L-arginine hydrochloride 6%; University Hospital Pharmacy, Erlangen, Germany) was administered i.v. at a dose of 100 mg/kg over 30 min. Over the following 30 min, L-arginine infusion was continued at 100 mg/kg over 30 min, but vitamin C (3 g; Cebion® forte; Merck, Darmstadt, Germany) was co-infused over the same time. Blood samples to determine inulin and p-aminohippurate concentration were drawn at 0, 120, 150 and 180 min. During the last 5 min of each infusion step, blood pressure was monitored every minute, and the mean of these measurements is given.

**Laboratory measurements**

Laboratory tests were performed at study inclusion to test for inclusion and exclusion criteria. Blood glucose concentration was measured in serum by use of the hexokinase reaction. On the day of the clearance study, relevant laboratory parameters were measured again. These parameters are displayed together with clinical data from the day of the clearance in Table 1. Measurement of p-aminohippurate and inulin was performed after completion of the study from blood samples centrifuged immediately at 4°C and stored at –21°C. p-Aminohippurate was measured by the method of Smith et al. [12]; inulin was determined indirectly with an enzymatic method following conversion to fructose. Each blood sample was measured in duplicate with a coefficient of variation of < 5%.

**Statistics**

Analyses were performed using SPSS Software (release 10.0; SPSS Inc., Chicago, IL) with paired and unpaired Student’s t-tests where appropriate. Mann–Whitney U-test was used for comparison of data not following the normal distribution. χ² test was used for comparison of gender differences between the groups. Adjustment for the effects of sex and age was performed by use of stepwise linear regression analysis. Data are given as mean ± SD. A P-value < 0.05 (two-sided) was considered to be significant.

**Results**

Baseline characteristics of the study cohort are given in Table 1. Except for differences in age (62 ± 5 vs 56 ± 5 years, P < 0.001) and diastolic blood pressure (85 ± 10 vs 93 ± 8 mmHg, P < 0.01), patients with type 2 diabetes and non-diabetic patients were well matched. In particular, renal haemodynamic parameters were not significantly different between the groups.

In patients with type 2 diabetes, RPF decreased in response to L-NMMA infusion (from 463 ± 121 to 409 ± 92 ml/min/1.73 m², P < 0.001), increased in response to L-arginine infusion (from 409 ± 92 to 454 ± 96 ml/min/1.73 m², P < 0.001) and increased further in response to co-infusion of vitamin C with L-arginine (from 454 ± 96 to 523 ± 119 ml/min/1.73 m², P < 0.001). In non-diabetic patients, RPF decreased in response to L-NMMA (from 433 ± 74 to 387 ± 66 ml/min/1.73 m², P < 0.001), increased in response to
Table 1. Baseline characteristics on the day of the clearance study

<table>
<thead>
<tr>
<th></th>
<th>Patients with type 2 diabetes (n = 20)</th>
<th>Patients without type 2 diabetes (n = 20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62 ± 5</td>
<td>56 ± 5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>16/4</td>
<td>12/8</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking status (former/never)</td>
<td>12/8</td>
<td>11/9</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>29.3 ± 2.9</td>
<td>26.4 ± 4.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Proportion of patients with arterial hypertension</td>
<td>17/20</td>
<td>14/20</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>152 ± 16</td>
<td>148 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>85 ± 10</td>
<td>93 ± 8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>212 ± 35</td>
<td>213 ± 44</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of type 2 diabetes (years)</td>
<td>14 ± 11</td>
<td>84 ± 14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>159 ± 55</td>
<td>84 ± 14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.4 ± 1.3</td>
<td>8.9 ± 0.18</td>
<td>NS</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.85 ± 0.20</td>
<td>1.8 ± 0.6a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Renal plasma flow (ml/min/1.73 m²)</td>
<td>463 ± 121</td>
<td>433 ± 74</td>
<td>NS</td>
</tr>
<tr>
<td>Glomerular filtration rate (ml/min/1.73 m²)</td>
<td>105 ± 13</td>
<td>102 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Filtration fraction (%)</td>
<td>24 ± 4</td>
<td>24 ± 4</td>
<td>NS</td>
</tr>
</tbody>
</table>

*aValues from the study inclusion laboratory are provided in control patients. HbA1c has not been measured in control patients.

The effects of L-NMMA and L-arginine on RPF were not different between patients with type 2 diabetes and non-diabetic patients (all P = NS). In contrast, the increase in RPF through vitamin C was more pronounced in patients with type 2 diabetes as compared with non-diabetic patients regardless of whether expressed in absolute terms (+47 ± 16 vs +43 ± 10 ml/min/1.73 m²; P < 0.05) or given as a percentage (+16 ± 10 vs +10 ± 8%, P = 0.07) (Figure 1). In a multiple stepwise linear regression, the presence or absence of type 2 diabetes was the only factor determining the response of RPF to vitamin C (R² = 0.113, β = 0.336, P = 0.037), whereas age (β = 0.059, P = 0.714) and sex (β = 0.022, P = 0.906) did not enter the final model. Data concerning other renal haemodynamic parameters are given in Table 2. No significant differences in the effects of L-NMMA, L-arginine and vitamin C on GFR and filtration fraction were observed between patients with type 2 diabetes and non-diabetic patients.

Data about the effects of L-NMMA, L-arginine and vitamin C on systemic haemodynamics are given in Table 2.

Discussion

In the current study, we found that L-NMMA-induced vasoconstriction of the renal vasculature was similar in hypertensive patients with and without type 2 diabetes. In contrast, co-infusion of vitamin C with L-arginine caused a more pronounced increase in renal perfusion in patients with type 2 diabetes than in non-diabetic patients.

Vasoconstriction in response to administration of the nitric oxide synthase inhibitor L-NMMA is used to assess nitric oxide bioavailability in vivo [13] and in vitro [14]. In accordance with this, the decrease in RPF in response to L-NMMA serves as a tool to analyse nitric oxide bioavailability in the renal vasculature [10,11]. The effect of L-NMMA on renal perfusion in both diabetic and non-diabetic patients is comparable with that observed in previous studies in middle-aged and elderly human subjects from our laboratory [10,15]. To outweigh L-NMMA-induced renal vasoconstriction, we infused L-arginine subsequent to L-NMMA infusion. This has been done on the basis of our previous finding that RPF returns to baseline values when L-NMMA (4.25 mg/kg) is followed by L-arginine at a dose of 100 mg/kg [10]. However, in this setting, the effect of L-arginine on renal haemodynamics should be interpreted with caution, since some of the pharmacological effects of L-NMMA might still be present despite this compound no longer being infused [10,16]. Therefore, we do not focus on the effect of L-arginine but use the achieved renal perfusion as a new baseline to examine the effect of vitamin C on renal haemodynamics.

The role of oxidative stress in the pathogenesis of diabetes has been examined repeatedly in experimental models. Renal cortical superoxide production is increased in the early stage of experimental diabetes leading to vasoconstriction of the afferent arteriole [6]. Altered L-NMMA-induced vasoconstriction and impaired acetylcholine-induced vasodilation of afferent and efferent arterioles can be restored by administration of superoxide dismutase mimetics [8,17]. Increased oxidative stress has been found in patients with type 2 diabetes as well [9,18]. In the forearm vasculature of patients with type 2 diabetes, endothelium-dependent vasodilation is improved by vitamin C [4]. There appears to be an association
between total antioxidant capacity and proteinuria [9], but this association was not found in another study [18]. However, the effect of antioxidant therapy on endothelium-dependent vasodilation in the renal circulation has not yet been assessed in patients with type 2 diabetes.

In our present study, co-infusion of vitamin C and L-arginine, which stimulates nitric oxide synthesis, caused a more pronounced increase in RPF in patients with type 2 diabetes than in non-diabetic control patients. In analogy to studies in the human forearm circulation [19,20], this finding indicates a higher amount of oxidative stress in the renal vasculature of patients with type 2 diabetes compared with non-diabetic patients. Nevertheless, the vitamin C-induced increase in RPF in diabetic subjects was smaller than
that observed in young smokers in a previous study [21]. Of note, active smokers were excluded from participation in the present study. It is likely that vitamin C infusion causes changes in glomerular haemodynamics by increasing nitric oxide bioavailability particularly in afferent glomerular arterioles. However, our present data are not sufficient to draw conclusions with regard to glomerular haemodynamics.

Under the conditions of our experimental protocol, it is unlikely that reactive oxygen species derive from a nitric oxide synthase-catalysed pathway due to substrate depletion, since L-arginine was co-infused with vitamin C [2]. However, it was not the aim of the present pilot study to examine the source of oxidative stress in the renal vasculature of patients with type 2 diabetes. Of note, differences in the response of renal perfusion to vitamin C infusion were observed despite the presence of antihypertensive treatment in the diabetic but not in the non-diabetic group.

In contrast to our experiments with vitamin C, we did not find a difference in the response to L-NMMA between patients with type 2 diabetes and non-diabetic patients. This suggests that nitric oxide bioavailability under basal conditions is similar between the two groups. Given an increased amount of oxidative stress that reduces nitric oxide bioavailability in patients with type 2 diabetes according to our vitamin C infusion study, one might suggest that basal nitric oxide production in the renal vasculature of patients with type 2 diabetes is even higher than in non-diabetic controls. This would, despite the increased oxidative stress, lead to similar basal nitric oxide bioavailability compared with non-diabetic patients. In accordance with this, nitric oxide synthase expression has been found increased in early stages of experimental diabetes [22]. Recently, Dalla Vestra and co-workers [23] found a reduction of RPF due to L-NMMA infusion in normoalbuminuric patients with type 2 diabetes and in non-diabetic controls, whereas RPF was not altered in microalbuminuric patients. Reduced nitric oxide synthesis in later stages of diabetic nephropathy might explain these results.

One might consider it a shortcoming of our study that we did not measure in vitro parameters of oxidative stress. However, we are convinced that in vivo studies such as ours that assess the net effect of the balance between a variety of vasoactive regulatory systems are at least equivalent if not superior to studies measuring markers that are not suitable to reflect the ‘true’ amount of oxidative stress [24]. Another limitation of our study is that it was not powered adequately to examine potential differences between normoalbuminuric and microalbuminuric patients. From the 20 patients included, microalbuminuria was present in six patients (data not shown). Although we did not find any significant differences between normo- and microalbuminuric patients in the response to infusions of experimental substances (data not shown), a greater number of patients is clearly warranted to draw definite conclusions. Finally, due to our inclusion criteria, we might have included patients at different stages of diabetic nephropathy. Further studies probably should use stricter inclusion criteria with regard to serum creatinine levels and albuminuria.

In summary, our findings of a similar nitric oxide bioavailability in hypertensive patients with and without type 2 diabetes but a more pronounced increase in renal perfusion by vitamin C in diabetic than in non-diabetic patients suggest that in the renal vasculature of patients with type 2 diabetes and with or without early diabetic nephropathy, oxidative stress is increased and basal nitric oxide production is also increased.

Acknowledgements. We gratefully acknowledge the technical assistance of Ortrun Alter. This study was supported by grants from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 423, TP B5) to C.D. and R.E.S.

Conflicts of interest statement. None declared.

References

3. Enderle MD, Benda N, Schmuelleing RM, Haering HU, Pfohl M. Preserved endothelial function in IDDM patients, but not in NIDDM patients, compared with healthy subjects. Diabetes Care 1998; 21: 1778–1779


Received for publication: 9.3.04
Accepted in revised form: 16.6.04