Analysis of NO-synthase expression and clinical risk factors in human diabetic nephropathy

Bernd Hohenstein¹, Christian P.M. Hugo¹, Birgit Hausknecht¹, Kirsten P. Boehmer¹, Regine H. Riess² and Roland E. Schmieder¹

¹From the Department of Nephrology and Hypertension, University Erlangen-Nuremberg, Erlangen and ²the Institute of Pathology, Klinikum Nuremberg, Nuremberg, Germany

Abstract

Background. Changes of renal nitric oxide (NO) production have been associated with glomerular hyperfiltration, vascular permeability, albuminuria, glomerulosclerosis and tubulointerstitial fibrosis. Several studies demonstrated an up- as well as downregulated expression of NO-synthases (NOS) in experimental diabetic nephropathy. It is still not yet specified whether the regulation and activity of NOS is changed in human diabetic nephropathy.

Methods. Renal biopsies and clinical data of 45 patients with diabetic nephropathy and of 10 control subjects were investigated. Glomerular and cortical endothelial NOS (eNOS) and inducible NOS (iNOS) expression were assessed by immunohistochemical staining and related to clinical data such as the duration of diabetes, insulin therapy and arterial hypertension, albuminuria/proteinuria, eGFR according to the formula modification of diet in renal disease (MDRD), presence of vascular complications or diabetic retinopathy.

Results. The mean age of patients at biopsy was 60.3 years and the mean duration of diabetes 12.9 years. Expression of cortical and glomerular eNOS was increased in type 2 diabetes ($P < 0.05$). Increased expression of glomerular and cortical eNOS correlated with more severe vascular complications ($r = 0.44; P < 0.05$). Glomerular eNOS was strongly increased among different degrees of proteinuria ($P < 0.01$). In contrast to expression levels of eNOS, the glomerular expression pattern of iNOS changed from an endothelial pattern in glomeruli with preserved morphology to expression predominantly by inflammatory cells.

Conclusions. Thus, increased eNOS expression by the renal endothelium could be demonstrated in type 2 diabetic nephropathy, whereas iNOS was unchanged but spatially differentially expressed. The eNOS expression was related to vascular lesions and the degree of proteinuria.

Keywords: diabetic nephropathy; eNOS; iNOS; type 2 diabetes

Introduction

Diabetic nephropathy is the major cause for end-stage renal failure in the Western world [1]. About 30–40% of all diabetic patients develop diabetic nephropathy putting them at high risk for end-stage renal disease and, in parallel, for severe micro- and macrovascular complications [2]. During early stages of diabetic nephropathy functional and structural abnormalities of glomerular capillaries occur. The earliest clinically detectable consequences are glomerular hyperfiltration [3,4] and the development of capillary leakage leading to microalbuminuria [5]. Among a variety of factors such as prostaglandins, atrial natriuretic peptide, glucagons, insulin and activation of protein kinases [6], nitric oxide (NO) is one of the major pivotal candidates that are believed to be involved in the early and late alterations of glomerular haemodynamics due to diabetes [7]. Interestingly, both decreased NO and increased NO activity have been found to cause endothelial dysfunction [8]. Most recently, it was observed that stimulation of the NO system can produce reactive oxygen species (ROS) if uncoupling of NO-synthases occurs, for example in the presence of low tetrahydrobiopterine availability [9]. Changes in the expression and function of the NO system related to diabetic nephropathy have been described by in vitro and in vivo experimental studies but to a much lesser extent by clinical studies in man [10].

There still exists controversy about the availability of NO during diabetic nephropathy and especially during very early disease stages. Previous experimental studies demonstrated down- as well as upregulation of the NO system in the kidney in various diabetic models [11–14]. These discrepant results might be related to the various methods applied to assess the availability of NO as well as to the fact that at least three NO-synthases, named neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible
NOS (iNOS), have been described and that these isoforms might be differentially altered in diabetic nephropathy [15]. Whereas eNOS and nNOS are considered as constitutively expressed, iNOS can be induced in consequence to various stimuli and are produced by almost all nucleated cells. Several studies have demonstrated the presence of all NOS isoforms within the human kidney [15–17]. In contrast, data on the expression and regulation of the NO system and NOSes during diabetic nephropathy in man are scarce [18–20].

In the present study, we investigated the eNOS and iNOS expression in kidney biopsies from patients with type 2 diabetic nephropathy. In addition, we related clinical and immunohistochemical data and could demonstrate that eNOS, but not iNOS expression, is stimulated in patients with type 2 diabetic nephropathy and linked to the degree of proteinuria, duration of insulin therapy and the presence of vascular complications.

Methods

Source of tissue

Archival tissues from core needle biopsies performed between 1992 and 2003 at the Klinikum Nuremberg (Nuremberg, Germany) were used for this study. In all patients renal biopsies had been performed due to suspected primary renal disease. The morphological diagnosis of diabetic nephropathy was made by the local pathologist unaware of study findings. Each diagnosis was confirmed by the past medical history of diabetes, thereby excluding patients with type 1 diabetes. Control tissues without evidence of renal disease (n = 10) were obtained from distant portions of kidneys surgically excised because of the presence of a localized neoplasm.

Tissue processing and immunohistochemical staining

All biopsies were fixed in 3% paraformaldehyde, embedded in paraffine and cut into 4 µm sections for indirect immunoperoxidase staining according to standard protocols. Negative controls for immunostaining included either deleting the primary antibody or substitution of the primary antibody with an equivalent concentration of an irrelevant rabbit mAb. A specific goat anti-rabbit HRP-conjugated secondary antibody was applied followed by colour development with AEC (3-amino-9-ethylcarbazole; DakoCytomation) a ready-to-use substrate chromogen.

To perform immunoperoxidase staining, tissue sections were incubated with the following primary and secondary antibodies, as indicated, at 4°C overnight.

Inducible NO-synthase was stained using AHP 303, a rabbit anti-mouse antiserum (Serotec, Duesseldorf, Germany) against iNOS without cross-reactivity against eNOS or nNOS.

AHP 302, a rabbit anti-mouse antiserum (Serotec, Duesseldorf, Germany) against endothelial eNOS without cross-reactivity for iNOS or nNOS was used for the detection of eNOS. All isoforms of NOS produce NO during a wide variety of physiological and pathophysiological processes from molecular oxygen [21].

Quantitative analysis of the immunostaining

For all stainings, all glomeruli were analysed in a blinded fashion using a 400-fold magnification; cortical areas were evaluated using a ×200 magnification. Glomerular and cortical expression of iNOS and eNOS were quantified using computer-assisted image analysis (MetaVue, Visitron Systems, Puchheim, Germany). For this evaluation, all areas (glomeruli and whole cortex) of the biopsy were photographed using a Leica DC-200 digital camera at a 400-fold magnification under standard lighting conditions (with respect to white balance and exposure). Using MetaVue software, image stacks were built and thresholds were set under visual control by the investigator to assure exact measurements. The same threshold was used for one image stack and—if necessary—adjusted for the next stack. For evaluation of glomeruli, glomeruli were selectively marked and measured, whereas for generation of cortical data the complete image (containing the tubulointerstitium) was measured. All biopsies had to have at least five glomeruli and the same number of cortical fields for evaluation. For every biopsy the local nephropathologist evaluated the severity of diabetic nephropathy in a blinded fashion. Thereby, each biopsy was semi-quantitatively graded according to the degree of glomerulosclerosis, tubulointerstitial fibrosis and inflammation from 1 to 3 as shown in Table 1, which describes the semi-quantitative grade of morphology in diabetic nephropathy. All findings were compared to renal biopsy specimen from kidneys with no evidence of renal disease (nrd). Representative images of grades 1 (1 A, B), 2 (1 C, D) and 3 (1 E, F) are depicted in Figure 1.

Clinical records

A total number of 45 renal biopsies were evaluated histologically and compared to 10 non-diabetic controls without evidence of renal disease and diabetes. Corresponding clinical data were retrieved from the clinical files and analysed. In 40 out of the 45 renal biopsies and in 10 of the 10 non-diabetic patients, parameters from clinical records were considered to be adequate since they had been assessed on the day of biopsy ±1 day. Of note, patients underwent renal biopsy if they were in stable clinical condition.

The following data were collected from the patients’ corresponding clinical records as present in the past medical history: age, duration of diabetes, duration of insulin therapy, presence of retinopathy (after diagnosis by ophthalmologist), presence of diabetic neuropathy (after diagnosis form a neurologist), presence of peripheral arterial occlusive disease including prior limb amputation due to arterial occlusive disease, presence of coronary heart disease, prior myocardial infarction, prior stroke, presence and duration of hypertension, medications inhibiting the renin–angiotensin system and history of (or current) smoking. Serological und urine testing including proteinuria, albuminuria, cholesterol levels, HbA1c concentrations, serum creatinine and creatinine clearance according to the
Table 1. Scoring system for the assessment of diabetic nephropathy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Grade 1 (n = 7)</th>
<th>Grade 2 (n = 7)</th>
<th>Grade 3 (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulosclerosis</td>
<td>Mildly increased glomerular matrix and sclerosis (up to 30%)</td>
<td>Moderate matrix expansion and sclerosis (&lt;50%)</td>
<td>Extensive matrix expansion, Kimmelstiel-Wilson lesions, global obliteration</td>
</tr>
<tr>
<td>Tubulointerstitial fibrosis</td>
<td>Mildly increased tubulointerstitial matrix</td>
<td>Increased matrix, initial tubular atrophy</td>
<td>Extensive matrix expansion and tubular atrophy</td>
</tr>
<tr>
<td>Inflammation</td>
<td>No relevant inflammatory reaction</td>
<td>Inflammation in up to 50% of the section</td>
<td>Severe inflammation in more than 50% of the tissue section</td>
</tr>
</tbody>
</table>

*n*: gives the number of biopsies per group.

Table 2. Baseline parameters of diabetic and non-diabetic patients and presence of diabetic complications

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diabetes group mean ± SD</th>
<th>Non-diabetic controls mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>60.3 ± 11.5</td>
<td>46.7 ± 22 n.s.</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>12.9 ± 8.0</td>
<td>0</td>
</tr>
<tr>
<td>Proteinuria (mg/dl)</td>
<td>4300 ± 5000</td>
<td>Not present</td>
</tr>
<tr>
<td>Albuminuria (mg/dl)</td>
<td>2008 ± 2501</td>
<td>Not present</td>
</tr>
<tr>
<td>Patients on insulin therapy (%)</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>Duration of insulin therapy (years)</td>
<td>6.6 ± 7.7</td>
<td>Not present</td>
</tr>
<tr>
<td>HbA1c level (mg/dl)</td>
<td>6.9 ± 1.7</td>
<td>Not present</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl; normal &lt; 1.2 mg/dl)</td>
<td>3.9 ± 2.8</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min; normal 90–140)</td>
<td>23.3 ± 21.4</td>
<td>68.6 ± 15.3</td>
</tr>
<tr>
<td>Serum urea (mg/dl; normal 10–50 mg/dl)</td>
<td>107 ± 74</td>
<td>31 ± 12</td>
</tr>
<tr>
<td>Patients with increased serum cholesterol (%)</td>
<td>71%</td>
<td>0</td>
</tr>
<tr>
<td>Presence of hypertension (%)</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Duration of hypertension (years)</td>
<td>9.3 ± 7.2</td>
<td>0</td>
</tr>
<tr>
<td>Patients on ACE inhibitors (%)</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>Prior history of stroke (%)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Prior history of myocardial infarction (%)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Presence of coronary heart disease (%)</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>Presence of arterial occlusive disease (%)</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>Prior history of ablation (%)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Presence of retinopathy (%)</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>Presence of diabetic neuropathy (%)</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>History of smoking (%)</td>
<td>65</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

modification of diet in renal disease (MDRD) equation were assessed during the hospital stay for renal biopsy. Hypercholesterolaemia was considered if total cholesterol levels were above 200 mg/dl and hypertension was defined according to the guidelines of the Deutsche Hochdruckliga and the European Society of Hypertension.

Since almost all patients were admitted to the hospital for renal biopsy, diagnoses had to rely on the patients’ past medical history, physical examination and serological measurements. To further quantify the degree of macrovascular complications, a vascular injury score was developed. The presence of peripheral arterial occlusive disease, coronary heart disease and prior myocardial infarction, prior stroke and prior limb amputation due to arterial occlusive disease were scored as 1 if positive and 0 if negative. Thereby, the presence of more severe vascular complications resulted in a higher score with a maximum score of 5. A complete score could only be calculated in 25 patients due to missing single parameters and diagnostic uncertainty from some patient records. All data were evaluated regarding differences dependent on the stage of disease and correlated to glomerular and cortical expression levels of iNOS and eNOS.

Statistical analysis

A Kolmogorov–Smirnov test was used to evaluate data distribution. Differences of NOS expression were tested using Student's t-test; all other comparisons were done using the non-parametric Mann–Whitney U-test. Data were presented as mean value ± SD. Statistical significance was defined as a P of < 0.05. Two-sided correlations were tested using Spearman's algorithm (SPSS 12.0, SPSS Software, Munich, Germany).

Results

Baseline characteristics of diabetic and non-diabetic patients

Clinical characteristics of groups and controls are given in Table 2. Diabetic patients had a mean age of 60.3 ± 11.5 years at renal biopsy compared to 46.7 ± 22 years of non-diabetic controls (not significant) and mean duration of diabetes was 12.9 ± 8.0 years. Most patients (78%) had proteinuria of >200 mg/dl (mean 4.3 ± 5 g/24 h), increased serum creatinine (89%; mean 3.9 ± 2.8 mg/dl) and
In kidney biopsies without any evidence of renal disease, expression of eNOS was virtually absent in glomerular endothelial cells, but positive in some mesangial (Figure 2A) as well as in tubular cells (Figure 2B). In diabetic nephropathy, eNOS expression was significantly enhanced in glomerular endothelial cells (Figure 2C, zoom), which represented the major source of eNOS. In glomeruli with enhanced glomerulosclerosis, glomerular endothelial cells still seemed to be the major source of eNOS expression; increased serum cholesterol levels could be detected in 71% of all patient records and 65% of our patients had a history of smoking.

eNOS is upregulated during type 2 diabetic nephropathy

In kidney biopsies without any evidence of renal disease, expression of eNOS was virtually absent in glomerular endothelial cells, but positive in some mesangial (Figure 2A) as well as in tubular cells (Figure 2B). In diabetic nephropathy, eNOS expression was significantly enhanced in glomerular endothelial cells (Figure 2C, zoom), which represented the major source of eNOS. In glomeruli with enhanced glomerulosclerosis, glomerular endothelial cells still seemed to be the major source of eNOS expression;
Fig. 2. Glomerular and cortical eNOS expression is increased during diabetic nephropathy. In kidney biopsies without evidence of renal disease, eNOS was widely absent in endothelial cells (A), whereas some mesangial (A), as well as tubular cells were positive for eNOS (B). During diabetic nephropathy, eNOS expression was significantly enhanced in glomerular endothelial cells (C, zoom, grade 1). By contrast, in more sclerotic glomeruli eNOS was predominantly expressed in areas of developing sclerosis and around Kimmelstiel–Wilson lesions (D, E, grade 3). Tubular eNOS expression hardly changed during diabetic nephropathy (F, grade 3) compared with kidneys without evidence of renal disease (B). Glomerular (G) as well as cortical (H) expression of eNOS was evaluated using computer-assisted image analysis after eNOS staining. Groups were built according to the pathological disease stages from 1 to 3 and for renal biopsies without evidence of disease (*P < 0.05).
however, as a consequence of disease progression and capillary loss, the distribution of these cells within the glomerulus changed (Figure 2D). In glomeruli with severe glomerulosclerosis, eNOS was also highly expressed in areas of developing sclerosis and around Kimmelstiel–Wilson lesions (Figure 2E). In addition, there was also positivity for eNOS next to injured capillaries that might originate from other cells such as mesangial or invading inflammatory cells (Figure 2E, zoom). In contrast, the tubular eNOS expression only slightly increased during diabetic nephropathy compared with kidneys without evidence of renal disease (Figure 2F).

Compared to kidneys with nrd (4.61 ± 3.3% positive area), the expression of glomerular eNOS was significantly increased during mild, moderate and severe diabetic nephropathy (grade 1: 15.6 ± 11.8%; grade 2: 9.65 ± 2.9%; grade 3: 14.1 ± 8.2% positive area; all P < 0.05). No differences between the different stages of disease could be found (Figure 2G). With respect to total cortical reflecting predominantly tubulointerstitial expression, a slight increase of eNOS expression was detected during diabetic nephropathy, which reached significance in biopsies with milder diabetic nephropathy (grade 1: 17.3 ± 9.6 versus 10.75 ± 5.5% positive area; P < 0.05; Figure 2H) most likely due to the small group size but not with more severe disease (grade 2 or 3).

Differential expression of iNOS during type 2 diabetic nephropathy

Since iNOS is a further potential source of NO within the diseased kidney, we also investigated the expression of glomerular and cortical iNOS. In control biopsies glomerular iNOS expression varied between complete absence (Figure 3A) and distinct expression in some mesangial and/or infiltrating cells (Figure 3B). In addition, several tubular cells demonstrated iNOS expression as shown in Figure 3A. In contrast, renal biopsies with diabetic nephropathy but nevertheless well-preserved glomerular morphology showed a discrete but selective, typical endothelial staining pattern for iNOS in glomerular capillaries (Figure 3C). Glomeruli with more enhanced diabetic lesions (Figure 3D, E) showed additional iNOS positivity of other most likely inflammatory cells and to a lesser extent of glomerular capillaries. This could be especially observed around developing Kimmelstiel–Wilson lesions, as shown by arrows in Figure 3D and E. The increased iNOS expression was also present in tubulointerstitial areas with inflammatory infiltrations (Figure 3F). Larger vessels of diabetic kidneys also demonstrated iNOS positivity of the endothelial layer as shown in Figure 3G.

Despite these differences in the expression pattern of iNOS, no differences regarding the glomerular (Figure 3H) or cortical (Figure 3I) expression level of iNOS could be detected within diabetic biopsies and compared to controls.

NOS expression relates to proteinuria, severity of vascular complications and duration of insulin therapy

Since NOS expression has been shown to correlate with diabetic complications such as albuminuria [18], we tested the correlation of clinical parameters with glomerular and cortical eNOS and iNOS expression in a subgroup of 40 patients with complete clinical records. Patients were divided into two groups according to the degree of proteinuria (> 1 g/day or < 1 g/day). In both groups, glomerular eNOS levels were clearly (P < 0.05) increased (< 1 g/day: 10.1 ± 4.1%; > 1 g/day: 15.9 ± 8.5% positive area) compared to controls (4.6 ± 3.3% positive area). Interestingly, glomerular eNOS was increased in patients with proteinuria of > 1 g/24 h (N = 24) compared to patients with proteinuria of < 1 g/24 h (N = 16) (15.9 ± 8.5% versus 10.1 ± 4.1% positive area; P < 0.01). For cortical eNOS, a strong tendency towards increased eNOS expression in patients with proteinuria of > 1 g/24 h was detected (12.4 ± 4.9 versus 17.7 ± 9.2; P = 0.06).

A score reflecting the severity of vascular complications correlated with the glomerular (1.9 ± 1.3; r = 0.44; P < 0.05) eNOS expression. On the day of biopsy, 25 of our patients were on insulin therapy and the duration of this therapy was related to NOS expression. Thereby, the degree of glomerular eNOS expression correlated with the duration of insulin therapy (r = 0.42; P < 0.05). Since the expression pattern of iNOS changed during the course of diabetic nephropathy, it seems interesting that glomerular iNOS expression also correlated with the duration of insulin therapy (r = 0.5; P < 0.05). No positive or negative correlations could be detected for any other parameters.

Discussion

In this study we investigated quantitative and qualitative expression patterns of the two isoenzymes, eNOS and iNOS, and their clinical relationship in type 2 diabetic patients with diabetic nephropathy using archival biopsies and the corresponding clinical data as assessed on the day of renal biopsy. Whereas eNOS was virtually absent in control glomeruli without evidence of renal disease but positive in some mesangial areas, glomeruli of diabetic nephropathy with a well-preserved morphology had an increased typical and prominent endothelial staining pattern. This pattern changed with ongoing disease and during ongoing injury eNOS expression was mainly detected in areas of developing sclerosis and around Kimmelstiel–Wilson lesions. In contrast, tubular cells demonstrated an intense and slightly increasing eNOS staining in diabetic biopsies. In addition, we also demonstrated the presence of iNOS in biopsies of diabetic kidneys throughout all disease stages. Whereas control biopsies had several iNOS positive tubular cells, iNOS expression in normal glomeruli varied between the complete absence and mesangial iNOS positivity. In contrast, diabetic glomeruli with a well-preserved morphology demonstrated an endothelial iNOS staining pattern (grade 1, Figure 3C). At later stages during diabetic nephropathy (stage 2 or 3), infiltrating mononuclear cells seemed to be the major iNOS source.

Our findings clearly strengthen the view that NO activity is stimulated during diabetic nephropathy. Despite some experimental results demonstrating either no or even decreased eNOS expression [12,13], many experimental diabetic models have also demonstrated an increase of eNOS.
Fig. 3. Glomerular and cortical iNOS is unchanged but differentially expressed during diabetic nephropathy. In glomeruli without evidence of renal disease we detected a spectrum of iNOS expression that varied between complete absence and positivity of few mesangial and/or infiltrating cells (A, B). Biopsies with diabetic lesions and a preserved glomerular morphology showed a typical endothelial staining pattern with iNOS-positive glomerular capillaries (C, grade 1). Glomeruli with more enhanced lesions (D, E, grade 3) showed additional iNOS positivity of other most likely inflammatory cells and to a lesser extent of glomerular capillaries, especially around developing Kimmelstiel–Wilson lesions (D, E). Expression of iNOS was also present in tubulointerstitial areas with inflammatory infiltrations (F, grade 3) and on the endothelial layer of larger vessels (G). Arrows indicate Kimmelstiel–Wilson lesions (D, E). Glomerular (H) as well as cortical (I) expression of iNOS was evaluated using computer-assisted image analysis. Groups were built according to the pathological disease stages from 1 to 3 and for renal biopsies without evidence of disease. No significant differences could be detected.
expression [11,14,22] by immunohistochemistry and at the mRNA level. Few but less detailed studies also indicated that this might also be the case for human diabetic nephropathy [18–20].

Many experimental studies have been performed to elucidate the role and regulation of the NO system in vivo regarding vascular reactivity, NO expression and modulation of haemodynamics that are the main changes during early diabetic nephropathy. However, results using different interventional strategies including specific and non-specific inhibitors of eNOS demonstrated positive as well as negative effects and remained controversial (reviewed in [15]).

Our finding of increased glomerular eNOS expression in a predominantly endothelial staining pattern goes along with findings by Hiragushi et al. [18] who demonstrated increased eNOS expression in diabetic patients with microalbuminuria and hyperfiltration. However, in contrast to their findings, we detected a relation between eNOS and the degree of proteinuria with a significant increase of glomerular eNOS expression with > 1 g proteinuria in 24 h. These findings are consistent with the view that increased eNOS might be a compensatory mechanism to endothelial damage [8] in type 2 diabetes by excess production of ROS [9] related to ineffective NO action [20]. However, our data cannot answer the question whether this increase is sufficient or not to prevent further endothelial injury.

Glomerular eNOS expression also correlated with the degree of vascular injury as assessed by a vascular injury score. This finding appears pathophysiologically very relevant considering the high frequency of vascular lesions and complications associated with the increased vascular morbidity of diabetic patients [23,24].

It appears that with advancing diabetic nephropathy the extent and degree of cardiovascular damage increases. Whereas we observed an increase of eNOS staining in the glomeruli, a decreased NO bioactivity is noted in the systemic circulation. Thus, while a compensatory upregulation is observed in the renal circulation, no compensatory up-regulation has been noted in the systemic circulation [25].

The role of iNOS during diabetic nephropathy is also quite unclear. Experimental studies have demonstrated the presence of iNOS in various cell types and it is proposed that it may contribute to increased NO generation, hyperfiltration and diabetic glomerular changes [26,27]; however, existing data are still quite controversially discussed [11,12,14,28]. To our knowledge, this is the first study to demonstrate the expression pattern of iNOS in human diabetic nephropathy. Whenever detected, data from experimental studies have found iNOS protein and mRNA to be unchanged [12–14]. The endothelial expression of iNOS in structurally preserved diabetic glomeruli and peritubular capillaries indicates that it might be induced due to early endothelial alterations and thereby contributes to the increased endothelial NO production during diabetic nephropathy (similar to eNOS). This initial expression pattern changed during later stages when iNOS expression mainly localizes to invading inflammatory cells rather than to glomerular cells.

The detected relationship between the duration of insulin therapy and the glomerular expression of iNOS and eNOS is a novel finding and may indicate just an association and not a causal relationship. Li et al. [4] demonstrated amelioration of glomerular haodynamic changes in diabetes by insulin therapy, which might be indirectly mediated via upregulation of NO expression and suggests a pathophysiological link regarding compensation of endothelial dysfunction in diabetic nephropathy. However, the direct link between insulin therapy and histology seems to be less reasonable than the link between the necessity of insulin treatment and a more protracted or complicated course of diabetes including the presence of comorbidities such as infections. Knowledge from functional experimental studies regarding iNOS is very limited and most studies reported short-term results such as the one by Veeken et al. who detected no effect of iNOS inhibition on early diabetic nephropathy [14]. In contrast, Trachtman and colleagues demonstrated that iNOS-deficient mice suffered from more severe diabetic lesions and faster disease progression [28] in a chronic diabetes model. Since patients with diabetic nephropathy are not routinely biopsied, the material for studies like ours is very rare and due to the long-lasting time course of disease, biopsies with very early lesions are even scarce. Considering that 75% of all biopsies were taken from patients with an estimated GFR of 50 ml/min or less, it becomes clear that even the morphologically best-preserved glomeruli or biopsies we used, still lack the histological information about early diabetic nephropathy and rather have to be compared with late or chronic effects in experimental models. In addition, these considerations have to be expanded towards control biopsies that cannot directly be compared with controls from experimental studies for the same reasons. Controls used for this study might have undergone certain changes with respect to local haemodynamics and as a consequence of reduced functional renal mass (with subsequent compensatory hypertrophy and changes of signalling molecules) due to the neoplasm. Ideal controls would be healthy kidneys or null biopsies from living kidney donors which are hard to come by.

Although our study has its limitations considering the number of patients and its retrospective design, human studies are rare and the number of patients studied is higher than most histological studies in patients with diabetic nephropathy. Thus, the present study demonstrated more detailed expression patterns of glomerular and cortical eNOS and iNOS during human diabetic nephropathy. Furthermore, the relationship between increased eNOS and the severity of diabetic vascular complications indicates the relevance to further investigate the NOS regulation as potential therapeutical target in diabetic nephropathy.

Acknowledgements. This study was supported by grants from the Deutsche Forschungsgemeinschaft KFG 106-2 and SFB 423 B5 to R.S. and SFB 423 B6 to C.H.

Conflict of interest statement. None declared.
References


Received for publication: 17.4.07
Accepted in revised form: 12.10.07