Role of iNOS and eNOS expression in a group of Egyptian diabetic and nondiabetic nephropathy patients

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Introduction and aim
Changes in renal nitric oxide (NO) production have been associated with glomerular hyperfiltration, vascular permeability, albuminuria, glomerulosclerosis, and tubulointerstitial fibrosis. This study aimed at detection of the role of both inducible nitric oxide synthases (iNOS) and endothelial nitric oxide synthases (eNOS) expression in diabetic and nondiabetic nephropathy patients.

Methodology
Renal biopsies and clinical data of 30 diabetic patients, 10 nondiabetic patients with renal impairment, and 10 control individuals were assessed for eNOS and iNOS expression.

Results
Both glomerular eNOS and iNOS expression levels were increased in diabetic nephropathy patients and this was associated with peripheral arterial occlusive disease. In nondiabetic patients, increased serum creatinine was found to be associated with increased iNOS and eNOS expression, and, together with the control group, they showed increased iNOS expression in tubular and interstitial cells. An association between cigarette smoking and increased expression of both iNOS and eNOS was detected in diabetic patients.

Conclusion
The presence of iNOS is associated with tubular damage resulting in renal failure. The upregulation of NO in diabetes mellitus type 2 may explain the endothelial dysfunction that is associated with almost all diabetic complications.

Keywords:
diabetic nephropathy, eNOS, iNOS, type 2 diabetes mellitus

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Introduction

Diabetic nephropathy is one of the most common and most severe microvascular complications of diabetes and is the leading cause of chronic and end-stage kidney disease worldwide [1]. Changes in the expression and function of the nitric oxide (NO) system related to diabetic nephropathy have been described by in-vitro and in-vivo experimental studies and to a much lesser extent by clinical studies in humans [2].

At least three nitric oxide synthases (NOSs) – neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) – have been described and these isoforms might be differentially altered in diabetic nephropathy [3]. Whereas eNOS and nNOS are considered as being constitutively expressed, iNOS can be induced in consequence to various stimuli and is produced by almost all nucleated cells. Several studies have demonstrated the presence of all NOS isoforms within the human kidney [4].

This study aimed at detecting the role of both iNOS and eNOS in kidney biopsies of Egyptian diabetic and nondiabetic nephropathy patients and at studying the clinical and immunohistochemical parameters associated with this expression.

Patients and methods

After being approved by the local scientific ethical committee and obtaining written informed consent from all participants, this cross-sectional study was conducted on 40 patients attending Kasr El Aini University Hospital. The patients were classified according to the presence of diabetes mellitus type II into group I, comprising 30 diabetic patients with a history of microvascular complications, and group II, comprising 10 nondiabetic patients with unexplained elevated creatinine levels above 3.5 mg/dl but who were not on dialysis. According to renal function, group I was further subdivided into group IA, comprising 10 patients with microalbuminuria and normal renal function, group IB, comprising 10 patients with macroalbuminuria and creatinine levels between 1.5 and 3 mg/dl, and group IC, comprising 10 patients with macroalbuminuria and creatinine levels above 3 mg/dl but who were not on dialysis. Ten patients suffering from a renal neoplasm

(group III), with no evidence of other renal diseases or diabetes mellitus, were included in our study to serve as the control group. Patients on renal dialysis were excluded from our study.

After a detailed assessment of medical history and a physical examination, all participants were subjected to biological tests and renal biopsies were obtained. For the sake of quantifying 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 as 0 if negative.

Renal biopsy

Renal biopsies were performed because of suspected primary renal disease for all patients of groups I and II, whereas the control tissue of group III without evidence of renal disease was obtained from distant portions of the kidneys surgically excised because of the presence of a localized neoplasm.

Detection of eNOS and iNOS in renal biopsies

The immunohistochemical reaction of renal biopsy samples obtained and stained with hematoxylin and eosin was evaluated using the avidin–biotin immunoperoxidase system (Dako, Carpentaria, California, USA). Pretreatment trypsinization and antigen retrieval of the sections were used to unmask the antigen and enhance the immunohistochemical reaction, as the formalin fixation may mask the tissue antigen and prevent its localization by the primary antibody because of excess aldehyde linkages.

The demonstration of antigen (iNOS and eNOS) in tissues and cells by immunostaining was carried out using three reagents:

(1) A primary antibody that reacted against a specific antigen and used to locate and bind to that antigen in the tissue.
(2) A linking antibody used to bind to the primary antibody, thus forming a bridge for the third, or labeling, antibody.
(3) A labeling antibody, by a peroxidase avidin–biotin immunoenzyme complex, to locate the antigens.

The entire antibody complex is made visible by addition of an appropriate substrate/chromogen reagent that is converted by the peroxidase label to a brownish precipitate at the site of antigen localization in the tissue.

NOS immunostaining

The primary iNOS and eNOS antibodies were used (Ab 1 : 50 for 60 min) for staining of formalin-fixed tissue requires boiling tissue section in 10 mm^3 citrate buffer, pH 6.0, for 10–20 min followed by coiling at room temperature for 20 min. Control: Tissue from the lung was used for the positive control. Cellular localization was cytoplasmic. Negative control: As a negative control for both markers, a renal tissue section was processed but the primary antibody was not added; instead, PBS was used. Negative control experiments were performed to evaluate kit selectivity. The microscope used for examining the collected processed cases was a Leitz-Diaplan (Wild Leitz GmbH, D-6330 Wetzlar, Germany).

Statistical analysis

The patients' data were analyzed using SPSS 17.0 (SSPS Inc., Chicago, Illinois, USA) for Windows 7. Quantitative variables are expressed as mean and SD and compared using an unpaired Student's *t*-test and the Mann–Whitney test. Qualitative variables are expressed as numbers (frequency) and percentages and compared between groups using the χ^2-test. *P*-values less than 0.05 were considered significant.

Results

The baseline data of our studied group are presented in Table 1. The present study showed that glomerular (cortical) expression of eNOS was nonsignificantly different in patients of groups IA, IB, and IC, although it was significantly expressed in patients of these groups compared with those of groups II and III. Although glomerular (cortical) expression of iNOS was nonsignificantly different in patients of groups IA, IB, and IC, it was significantly expressed in patients of groups IB and IC compared with those of group III; iNOS was significantly expressed in patients of group II compared with those of group IA (Table 2). Staging of diabetic nephropathy was made in the studied diabetic patients of group I and the results are presented in Table 2. There was no statistically significant difference in eNOS or iNOS expression among the different stages of diabetic nephropathy, nor was there a statistically significant difference between the eNOS and iNOS glomerular, tubular, or interstitial expression among the three stages of diabetic nephropathy. Although patients of group II showed a significant marked expression of both eNOS and iNOS compared with those in group III ($P = 0.04$, for both), there was no statistically significant difference in their glomerular expression ($P = 0.1$ and 0.2, respectively). In patients of group I with diabetic nephropathy the presence of proteinuria did not significantly influence eNOS or iNOS expression ($P = 0.5$ and 0.2, respectively), whereas the presence of high serum creatinine level above 2 mg/dl did (Table 3). Moreover, the duration of insulin therapy in patients of group I did not influence their expression of both eNOS and iNOS ($P = 0.3$ and 0.6, respectively). With regard to the diabetes mellitus complications in group I and their influence on both eNOS and iNOS expression, it was reported that the presence of peripheral arterial occlusive disease and hypertension was significantly associated with increased expression of eNOS and iNOS (with *P*-value 0.04 and 0.03, respectively). Chronic heavy smoking significantly influenced both eNOS and iNOS expression in diabetic

Table 1 Data of the studied groups

Parameters	Group I			Group II	Group III
	Group IA	Group IB	Group IC		
Age (years)	52.1 ± 3.5	55.8 ± 5.6	52.8 ± 5.9	36.5 ± 5.9	52.9 ± 8.2
Proteinuria (mg/dl)	0.2 ± 0.08	1.2 ± 0.6	1.5 ± 0.6	0.6 ± 0.8	–
Albuminuria (mg/dl)	93.7 ± 25.1	45 ± 11.9	30.6 ± 13.1	1.2 ± 0.6	–
Duration of insulin therapy (years)	4.7 ± 3	5.7 ± 2.8	7.3 ± 2.7	–	–
Serum creatinine (mg/dl)	0.9 ± 0.4	2.2 ± 0.5	5.1 ± 1.2	5.7 ± 1.6	0.8 ± 0.3
Creatinine clearance (ml/min)	60 ± 55	40 ± 35	25 ± 32	30.9 ± 17.8	102.2 ± 21.2
Serum urea (mg/dl)	28.5 ± 11.3	98 ± 31.2	171.3 ± 46.9	171 ± 43.7	29.3 ± 10
Patients with increase serum cholesterol	163.5 ± 49.1	198.2 ± 32.3	216 ± 24.9	175 ± 47.4	164.9 ± 41
History of smoking (%)	60	50	63	10	10

Table 2 Comparison between glomerular (cortical) eNOS and iNOS expression of the studied group of patients

	eNOS (negative/positive)	iNOS (negative/positive)
Group IA [*N* (%)]	1/9 (10/90)	8/2 (80/20)
Group IB [*N* (%)]	1/9 (10/90)	4/6 (40/60)
Group IC [*N* (%)]	0/10 (0/100)	3/7 (30/70)
P-value	0.1	0.06
Group II [*N* (%)]	8/2 (80/20)	3/7 (30/70)
P-value		
Group IA vs. Group II	0.001	0.04
Group IB vs. Group II	0.001	0.1
Group IC vs. Group II	0.002	0.1
Group III [*N* (%)]	10/0 (100/0)	7/3 (70/30)
P-value		
Group IA vs. Group III	0.0001	0.3
Group IB vs. Group III	0.0001	0.01
Group IC vs. Group III	0.0001	0.02

eNOS, endothelial nitric oxide synthases; iNOS, inducible nitric oxide synthases.

Table 3 Comparison between expression of eNOS and iNOS in all diabetic patients included in the study in terms of serum creatinine and proteinuria

	N (%)		
	eNOS (negative/positive)	iNOS (negative/positive)	*P*-value
Creatinine (mg/dl)			
<2	3/7 (30/70)	4/6 (40/60)	0.01
>2	0/20 (0/100)	1/19 (5/95)	0.02
Proteinuria			
<1	0/14 (0/100)	8/6 (57.14/42.58)	0.5
≥1	1/15 (6.25/93.37)	6/10 (37.5/62.5)	0.2

eNOS, endothelial nitric oxide synthases; iNOS, inducible nitric oxide synthases.

patients of group I ($P = 0.9$ and 0.7, respectively, which were nonsignificant) (Table 4).

Table 4 Comparison between expression of eNOS and iNOS in all diabetic patients included in the study and effect of Peripheral arterial occlusive disease, hypertension and smoking.

	Group IA		Group IB		Group IC		
	N	%	*N*	%	*N*	%	*P*-value
Peripheral Arterial Occlusive Disease							
eNOS negative/positive	8/2	80/20	7/3	70/30	3/7	30/70	0.05
iNOS negative/positive	8/2	80/20	5/5	50/50	4/6	40/60	0.04
Hypertenion							
eNOS negative/positive	5/5	50/50	3/7	30/70	0/10	0/100	0.04
iNOS negative/positive	4/6	40/60	2/8	20/80	0/10	0/100	0.03
Smoking							
eNOS negative/positive	4/6	40/60	4/6	40/60	5/5	50/50	0.9
iNOS negative/positive	3/7	30/70	5/5	50/50	5/5	40/50	0.7

eNOS, endothelial nitric oxide synthases; iNOS, inducible nitric oxide synthases.

Discussion

During early stages of diabetic nephropathy, functional and structural abnormalities of glomerular capillaries are observed. The earliest clinically detectable consequences are glomerular hyperfiltration, followed by development of capillary leakage leading to microalbuminuria [5]. Interestingly, both decreased NO and increased NOS activity have been found to cause endothelial dysfunction [6]. There still exists controversy about the availability of NO during diabetic nephropathy and especially during very early stages of disease. Previous experimental studies have shown discrepant results of NO regulation in the kidney in various diabetic models [7]. In this study it was found that eNOS expression was virtually absent in control glomeruli without evidence of renal disease, contrary to glomeruli with evidence of diabetic nephropathy, which showed intense eNOS positivity in different selected diabetic groups. eNOS was released from the endothelium of afferent arterioles, glomeruli, and efferent arterioles; thus, its intense expression in diabetic patients may give the impression that it has a role in endothelial dysfunction seen in diabetic patients.

The expression of iNOS in both controls and diabetic patients may be explained by the following events. Our control patients are not diabetic and do not have renal impairment; however, they were originally diagnosed with a neoplasm. Thus, the controls used for this study might have undergone certain changes with respect to local hemodynamics and as a consequence of reduced functional renal mass, with subsequent hypertrophy and changes in signaling molecules, due to the neoplasm; however, our sample was too small (10 patients only) and the iNOS expression was not statistically significant. In

contrast, diabetic glomeruli with preserved morphology demonstrated an endothelial iNOS staining pattern. At later stages during diabetic nephropathy, infiltrating mononuclear cells seemed to be the major NO source. Expression of iNOS and eNOS in diabetes may be because of the metabolic changes of diabetes associated with NO production, as well as because of endothelial dysfunction, which is responsible for all complications of diabetes [8].

eNOS has a protective vascular effect, whereas iNOS can cause tubular damage [9]. In the nondiabetic patients with renal impairment and in the control individuals in this study, eNOS expression varied from complete absence to its presence in a few samples. In contrast, iNOS showed marked positivity in nondiabetic patients with renal impairment compared with the control group, giving the impression that the presence of iNOS with marked positivity may play a role in the development of renal failure. However, because of the small number of patients selected we do not have confirmatory data pertaining to the role of eNOS.

Expression of iNOS increases with the presence of diabetes, but eNOS starts to be actively expressed in early diabetic nephropathy [10]. The extent of expression of both eNOS and iNOS in different studied diabetic groups with progression of diabetic nephropathy showed minimal change in the expression of glomerular eNOS, whereas iNOS was positively expressed with the progression of nephropathy. With the progression of diabetic nephropathy there are more inflammatory cells that are the major source of NO, especially of iNOS, which may explain our finding.

In the diabetic group studied in a previous study, eNOS was expressed in glomerular endothelial cells with some expression in the tubular epithelium and interstitial cells, which may be attributed to the endothelial dysfunction seen in diabetic patients, whereas in the nondiabetic renal impairment patients it was expressed at the tubular and interstitial levels, explaining the role of NO in tubular injury and the development of renal failure, while being completely absent in normal glomeruli with some positivity in tubular and interstitial cells in the control group. With regard to the iNOS expression in the diabetic group and the nondiabetic renal impairment group it was highly expressed in tubular and interstitial cells, whereas in the control group it varied from complete absence to its presence in some endothelial cells with intense expression in tubular and interstitial cells, which emphasizes the finding that NO activity is stimulated during diabetic nephropathy [7].

In comparison with another study, our results could not emphasize the finding that the expression of NOS correlates with diabetic complications [7] because of the small sample size of our studied group. Some patients included in our study with renal impairment have hypertension. This may be because of glomerular iNOS deficiency associated with endothelial dysfunction that progressed to atherosclerosis, hypertension, and renal failure [11].

In type II diabetes, smoking increases the risk of developing microalbuminuria [12]. In our study there was an association between smoking and expression of both iNOS and eNOS in diabetic patients, which may explain the role of smoking in endothelial function.

Conclusion

In this study, diabetic nephropathy was seen to be associated with increased iNOS and eNOS expression.

(1) Endothelial dysfunction is the cause of all complications in diabetic patients and in nondiabetic patients with renal impairment.
(2) iNOS in diabetic patients with renal failure may explain the pathophysiology responsible for renal failure.
(3) The marked expression of NO in diabetic patients more than in other groups may suggest that the presence of diabetes adds to the complications seen in patients with diabetes either with or without renal failure.

Recommendation

A prospective study on a larger sample size to emphasize the significance of our results.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Remuzzi G, Schieppati A, Ruggenenti P. Clinical practice. Nephropathy in patients with type 2 diabetes. N Engl J Med 2002; 346:1145–1151.
2 Delles C, Klingbeil AU, Schneider MP. The role of the nitric oxide in the regulation of glomerular hemodynamics in humans. Nephrol Dial Transplant 2004; 19:1392–1397.
3 Komer R, Andreson S. Paradoxes of nitric oxide in the diabetic kidney. Am J Physiol Renal Physiol 2003; 284:F1121–F1137.
4 Heeringa P, Van Goor H, Itoh-Lindstrom Y. Lack of endothelial nitric oxide synthase aggrevates murine accelerated anti-glomerular basement membrane glomerulonephritis. Am J Pathol 2000; 156:879–888.
5 Wolf G, Ziyadeh FN. Molecular mechanisms of diabetic renal hypertrophy. Kidney Int 1999; 56:393–405.
6 Goligorsky MS. Endothelial cell dysfunction and nitric oxide synthase. Kidney Int 2000; 58:1360–1376.
7 Schwartz D, Schwartz IF, Blantz RC. An analysis of renal nitric oxide contribution to hyperfiltration in diabetic rats. J Lab Clin Med 2001; 137:107–114.
8 Prabhakar SS. Role of nitric oxide in diabetic nephropathy. Semin Nephrol 2004; 24:333–344.
9 Schrier RW, Wang W, Poole B, Mitra A. Acute renal failure definitions, diagnosis, pathogenesis, and therapy. J Clin Invest 2004; 114:5–14.
10 Hohenstein B, Hausknecht B, Boehmer K, Riess R, Brekken RA, Hugo CP. Local VEGF activity but not VEGF expression is tightly regulated during diabetic nephropathy in man. Kidney Int 2006; 69:1654–1661.
11 Nakayama T, Sato W, Kosugi T, Zhang L, Campbell-Thompson M Yoshimura A, *et al*. Endothelial injury due to eNOS deficiency accelerates the progression of chronic renal disease in the mouse. Am J physiol Renal Physiol 2008; 52:154–178.
12 Biesenbach G, Grafinger P, Janko O. Influence of cigarette smoking on the progression of clinical diabetic nephropathy in type 2 diabetic patients. Clin Nephrol 1997; 48:146–150.