Full Length Research

Oxidative stress and not endothelial Nitric Oxide Synthase gene polymorphism involved in diabetic nephropathy

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Diabetic Nephropathy (DN) poses a major health problem. There is strong evidence for a potential role of the eNOS gene. The aim of this case control study was to investigate the possible role of genetic variants of the endothelial Nitric Oxide Synthase (eNOS) gene and oxidative stress in the pathogenesis of nephropathy in patients with diabetes mellitus. The study included 124 diabetic patients; 68 of these patients had no diabetic nephropathy (group 1) while 56 patients exhibited symptoms of diabetic nephropathy (group 2). Sixty two healthy non-diabetic individuals were also included as a control group. Blood samples from subjects and controls were analyzed to investigate the eNOS genotypes and to estimate the lipid profile and markers of oxidative stress such as malondialdehyde (MDA) and nitric oxide (NO). No significant differences were found in the frequency of eNOS genotypes between diabetic patients (either in group 1 or group 2) and controls (p >0.05). Also, no significant differences were found in the frequency of eNOS genotypes between group 1 and group 2 (p >0.05). Both group 1 and group had significantly higher levels of nitrite and MDA when compared with controls (all p = 0.0001). Also group 2 patients had significantly higher levels of nitrite and MDA when compared with group 1 (p = 0.02, p = 0.001 respectively). The higher serum level of the markers of oxidative stress in diabetic patients particularly those with diabetic nephropathy suggest that oxidative stress and not the eNOS gene polymorphism is involved in the pathogenesis of the diabetic nephropathy in this subset of patients.

Key words: eNOS polymorphism, oxidative stress, diabetic nephropathy.

INTRODUCTION

Diabetic nephropathy (DN) is one of the microvascular complications of diabetes mellitus (DM) and one of the leading causes of death among these patients (Chiarelli et al., 2009). It begins with microalbuminuria and progresses to overt proteinuria and continues to deteriorate until end stage renal disease (Brenner et al., 2004). About one-third of type 1 or type 2 diabetic patients develop DN, even with proper blood glucose control. Furthermore, there is strong evidence for a genetic role in DN (Mogyorosi and Ziyadeh, 2001).

Endothelial nitric oxide synthase (eNOS) is a constitutively expressed enzyme of 135 kDa in the vascular endothelial cells. The eNOS gene, located on chromosome 7q35-36, is composed of 26 exons and spans 21 kb. A commonly reported polymorphism of the eNOS gene is the 27 bp deletion (allele a) and insertion

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(allele b) polymorphism in intron 4. Since the I/D polymorphism is an intronic marker, it may be functionally neutral but is in strong linkage disequilibrium with another un-observed functional mutations in the eNOS gene. The 4a allele has been reported to be associated with a number of phenotypes including hypertension among patients with type 2 diabetes mellitus (Pulkkinen et al., 2000), DN (Zanchi et al., 2000) and terminal renal disease (Buraczynska et al., 2004).

Nitric oxide (NO) is synthesized continuously in the endothelium from L-arginine by eNOS. It plays an important role in maintaining basal vascular tone through its effect on the soluble Guanylate Cyclase signaling pathway (Moncada et al., 1993). It also inhibits platelet as well as leukocyte adhesion to vascular endothelium and inhibits proliferation of smooth muscle cells via a Guanylate Cyclase-independent mechanism (Kubes et al., 1991). It has been demonstrated both *in vitro* and *in vivo* that overproduction of NO may induce oxidative stress in retinal, endothelial, and glomerular cells (Shibuki et al., 2000).

Three eNOS polymorphisms comprising the −786T/C single nucleotide polymorphism (SNP) in the promoter region, the G894T missense mutation in exon 7 and the intron 4 27-bp repeat (4b4a) were associated both with coronary artery disease (CAD) and nephropathy (Nakayama et al., 1999). However, the potential link between eNOS gene variants with the induction and progression of DN has provided contradictory results as shown by the association of the G894T SNP with end-stage-renal disease (ESRD) and DN by some studies (Noiri et al., 2002) but denied by other studies (Liu et al., 2005). Variable association was reported between the 4a allele, which is linked with reduced serum NO concentrations and endothelial dysfunction in type 2 diabetes mellitus (Komatsu et al., 2002) and DN (Negebauer et al., 2000).

It has been postulated that localized tissue oxidative stress is a key component in the development of DN. However, there is some controversy as to whether it represents an early link between hyperglycemia and renal disease or it develops as a consequence of other primary pathogenic mechanisms. In the kidney, a number of pathways generate reactive oxygen species (ROS) such as glycolysis, specific defects in the polyol pathway, uncoupling of nitric oxide synthase, xanthine oxidase, NAD(P)H oxidase and advanced glycation have been identified as major contributors to the pathogenesis of diabetic kidney disease (Thum et al., 2007).

Increased production of ROS in diabetes may be one of the common mechanisms of diabetic vascular compli-cations, including nephropathy. So, assessment of the oxidative stress production is important for the prediction and prevention of diabetes-related complications (Satoh et al., 2005). The aim of the present study was to investigate the possible role of the genetic variants eNOS gene and oxidative stress in the pathogenesis of

nephropathy in patients with DM.

METHODOLOGY

Clinical methods

The current study included 124 diabetic patients. Patients were recruited from the Diabetic Out-patient Clinic of the Specialized Medical Hospital, Mansoura University, Egypt. Sixty two healthy adults without diabetes were selected on voluntary bases and comprised the control group. Local Ethics Committee approval and written consent was obtained from the patients and the controls. The patients were classified according to the progress of the disease into 68 patients without nephropathy (group 1) and 56 patients with diabetic nephropathy (group 2). Diagnosis of diabetes was based on the criteria established by the American Diabetes Association Expert Committee [fasting serum glucose concentration >126 mg/dl (7.0 mmol/l) or a 2 h post load value in the OGTT >200 mg/dl (11.1 mmol/l) on more than one occasion] (Rotimi et al., 2001). DN was defined as urinary albumin excretion of >30 mg in 24 h urine collections (Orsonneau et al., 1989). Patients with collagen vascular diseases, chronic infections, liver diseases as well as those with kidney diseases other than DN were excluded from the study.

Laboratory methods

All the subjects and controls were required to provide a complete medical history and were given a thorough clinical examination. Body Mass Index (BMI) was assessed from weight in kilograms divided by height in meters squared. Mean systolic and diastolic blood pressure were measured according to a standardized protocol after at least 10 min rest using a validated automated machine and appropriate cuff. Direct Fundoscopy was performed after the pupils were dilated with tropicamide.

Five milliliter blood samples from the subjects and 1 mL blood samples from the control were acquired in the fasting state. The blood samples were stored in EDTA-coated tubes and were used for the estimation of glycosylated hemoglobin (HbA_{1c}) and extraction and analysis of DNA (Abraham et al., 1978). Serum samples were obtained from 4 ml blood samples for the estimation of serum nitrite and MDA levels. Also fasting blood glucose (Trinder, 1969), total cholesterol (Allain et al., 1974), triglyceride (Fossati and Prencipe, 1982), low density lipoprotein-cholesterol (LDL-cholesterol), high density lipoprotein-cholesterol (Warnick and Wood, 1995) and creatinine (Murray, 1984) were measured. In addition, 24 h urine samples were collected for protein analysis (Orsonneau et al., 1989).

Extraction of DNA and use of RFLP for detection of polymorphisms

Genomic DNA was extracted from peripheral blood using Gentra Genomic DNA purification kit. The DNA provided by the extraction containing the eNOS gene was amplified by Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, Norwalk, CT., U.S.A.). A set of primers were designed to amplify a 206 bp fragment including the missense G894T polymorphism (Glu298Asp variant) [5'-CAT GAG GCT CAG CCC CAG-3' (forward) and 5'-AGT CAA TCC CTT TGG TGC TCA C-3'(reverse)]. Each PCR was performed using 300 ng of DNA, 200 mmol/L of each dNTP, 500 nmol/L of each primer and 2.5 units of Taq DNA polymerase. DNA was initially denatured for one minute at 95°C, and then, 35 cycles were performed as
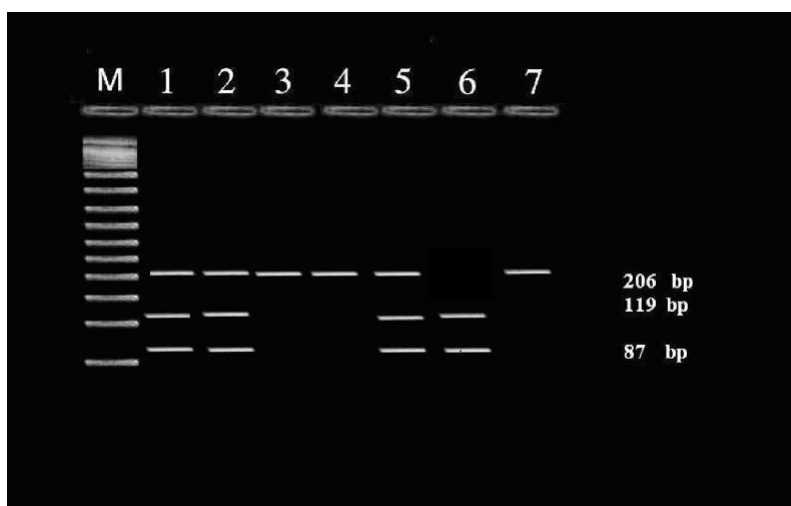
Figure 1. Ethidium bromide stained 3% agarose gel shows enzymatic digestion of G894T polymorphism of eNOS gene for the different groups studied; lane M: 50 bp DNA size marker in bp labeled from bottom up (50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800), lane 1,2 and 5 (GT genotype), lane 3,4 and 7 (GG genotype), lane 6(TT genotype).

follows; 95°C for 25 s, 56°C for 35 s and 72°C for 40 s. The PCR amplification was completed by a final extension at 72°C for 7 min. The amplification yielded a product of 206 bp which was subjected to RFLP. Upon cleavage by 5 units of *Mbo*I restriction enzyme (Boehringer Mannhiem) for 16 h at 37°C, the product was subjected to electrophoresis on 3% agarose gel, stained with ethidium bromide, and visualized under UV light. Allele T produced 2 bands at 119 bp and 87 bp whereas the G allele remained un-cut. Three patterns were observed during electrophoresis: (a) a 206 bp fragment which was produced by the normal genotype GG, (b) 206, 119, 87 bp fragments which were produced by heterozygous mutated genotype GT, and (c) 119, 86 bp fragments which were produced by homozygous mutated genotype TT, as can be seen for Figure 1.

Determination of malondialdehyde (MDA)

Serum proteins were precipitated by addition of tricholoroacetic acid and lipid peroxidation product, malondialdehyde (MDA), was measured by thiobarbituric acid assay, which is based on MDA reaction with thiobarbituric acid to give Thiobarbituric acid reactive substances (TBARS) which were measured by UV spectroscopy at 532 nm (Draper and Hardley, 1990).

Determination of serum nitric oxide (NO)

Serum nitrite levels were estimated by colorimetric assay which is based on the Griess reaction using a two-step diazotization reaction in which acidified nitrite produces a nitrosating agent that reacts with sulfa nitric acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylene diamine dihydrocholoride to form a red azo derivative which can be detected by UV-Vis. spectroscopy at 540 nm (Green et al., 1982).

Statistical analysis

Statistical analysis was performed by the use of MedCalc® program version 8.1.0.0 (Schoonjans et al., 1995). The parametric data was expressed as mean and standard deviation. The non-parametric data was expressed as median and range. Mann-Whitney test was used as a test of significance for comparison of two groups. Spearman rank correlation coefficient was done to study the relation between variables. P value was considered significant if less than 0.05.

RESULTS

This study comprised 124 diabetic patients; 68 of these patients showed no symptoms of diabetic nephropathy (group 1) while 56 patients had symptoms of diabetic nephropathy (group 2). Sixty two healthy non-diabetic individuals were also included as a control group. Such a sampling size is valid for ethnically homogenous groups such as Egyptian or Japanese populations. Previously, a Japanese study on Diabetic Nephropathy has been published (Neugebauer et al., 2000). Its sampling size consisted of 82 types 2 diabetic patients without nephropathy, 94 patients with microalbuminuria, 39 patients with Diabetic Nephropathy, and 155 healthy control subjects.

Table 1 shows the characteristics of the subjects and the control. The mean age of diabetic patients without nephropathy (group 1) was 46.6 ± 13.2 which ranged from 17 to 64 years, for diabetic patients with nephropathy (group 2) it was 39.8 ± 11.5 which ranged from 19 to 69 years and that of the control 36.4 ± 8.04 which ranged from 25 to 50 years. Serum levels of TG, total cholesterol, LDL-C were significanty higher in groups 1 and 2 patients as compared to controls (all p<0.0001), but no significant differences were found

Table 1. Characteristics of the subjects and controls.

Parameter	Control (n = 62)	Group 1 (n = 68	Group 2 (n = 56)
Age (years)	36.4 ± 8.04 (25–50)	46.6 ± 13.2 (17–64)	39.8 ± 11.5(19–69)
M:F	30/32	33/35	25/31
Age at onset of diabetes (years)		38.2 ± 9.2 (31–52)	41.3 ± 11.0(27–54)
Duration of diabetes (years)		19.4 ± 8.2 (14–31)	18.6 ± 8.1 (13–34)
Insulin dosage (units)		58.2 ± 27.5(20–118)	43.8 ± 15.7 (25–94)

Table 2. Comparison of the biochemical parameters between the studied groups.

Parameter	Control (n = 62)	Group 1 (n = 68)	Group 2 (n = 56)	p-value
TG (mg/dl)	124.2 ±10.89	151.4 ±5.28	152.5±4.59	P1<0.0001 P2<0.0001 P3 = 0.2
Total cholesterol (mg/dl)	171.4 ±6.14	193.5 ± 4.16	194.6±4.30	P1<0.0001 P2<0.0001 P3 = 0.1
LDL-c (mg/dl)	92.70 ± 4.08	110.80±4.72	112.15±3.84	P1<0.0001 P2<0.0001 P3=0.08
HbA1c (mg/dl)	5.9± 1.59	6.3± 2.49	6.1± 1.59	P1 = 0.8 P2 = 0.4 P3 = 0.6

P1: Group1 Vs control, P2: group 2 Vs control, P3: group 1 Vs group 2. p ≤ 0.05 is significant.

between group 1 and 2 patients (p>0.05). No significant differences were found between both groups of patients and control or between group 1 and 2 patients as regard HbA_{1c} (p>0.05) (Table 2).

The results of PCR determination of G894T genotypes of eNOS in the studied groups revealed no significant differences in genotypes frequency(GG, GT and TT) between groups 1 and 2 patients when compared with controls (P > 0.05). No significant differences in these genotypes were found between group 1 and group 2 (P > 0.05). Also, no significant differences were found in allele's frequency (*T and G alleles*) between groups 1 and 2 patients when compared with controls (P > 0.05) (Table 3).

Significantly higher serum levels of of nitrite and MDA were found in groups 1 and 2 patients when compared with controls (all P<0.0001). Also, significantly higher serum levels of of nitrite and MDA were found in group 1 when compared with group 2 patients (p = 0.02 and 0.001 respectively) (Table 4). Comparison of the serum levels of of nitrite and MDA between the different G894T genotypes of eNOS proved significantly higher serum levels of nitrite in TT genotype when compared with GG

genotype (p = 0.002), also, serum level of MDA was significantly higher in TT and GT genotypes when compared with GG genotype (p = 0.02, p = 0.03 respectively) (Table 5).

Figure 5 shows the fragment sizes from G894T polymorphism of eNOS gene after cleavage by Mbo I restriction enzyme are as follows: lane 1, 2 and 5 the heterozygous GT genotype, lane 3, 4 and 7 the homozygous GG genotype, lane 6 the homozygous TT genotype).

DISCUSSION

There is strong evidence suggesting a genetic basis for DN. The candidate genes include Endothelial Nitric Oxide Synthase (eNOS) gene. Endothelial Nitric Oxide Synthase is an enzyme that is important for the regulation of blood flow and blood pressure. Any defect in this enzyme can lead to diabetic nephropathy and/or retinopathy (Ismail et al., 1999).

Oxidative stress is also viewed as playing a critical role in the pathogenesis of both the micro- and macrovascular

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Table 3. Comparison of the frequencies of G894T genotypes of eNOS gene between the studied groups.

Genotypes	Control (n = 62)	Group 1 (n = 68)	Group 2 (n = 56)	p-value
GG	41(66.1%)	52(76.5%)	36(64.3%)	P1 = 0.26 P2 = 0.99 P3 = 0.19
GT	16(25.8%)	10(14.7%)	12(21.4%)	P1 = 0.17 P2 = 0.73 P3 = 0.46
TT	5(8.1%)	6(8.8%)	8(14.3%)	P1 = 0.86 P2 = 0.43 P3 = 0.49
G allele	98(79%)	144(83.8%)	84(75 %)	P1 = 0.4 P2 = 0.56 P3 = 0.11
T allele	26(21%)	22(16.2%)	28(25%)	P1 = 0.4 P2 = 0.56 P3 = 0.11

Table 4. Comparison of serum nitrite (µmol∕ml) and MDA (nmol∕ml) levels of the studied groups.

Parameter	Control (n = 62)	Group 1(n = 68)	Group 2 (n = 56)	p-value
Nitrite (µmol∕ml)	13.49±4.1	17.41±5	19.11±3.2	P1<0.0001 P2<0.0011 P3= 0.02
MDA (nmol∕ml)	1.33±0.35	2.28±0.28	2.55±0.44	P1<0.0001 P2 = 0.0001 P3 = 0.001

Table 5. Serum nitrite (µmol∕ml) and MDA (nmol∕ml) levels among different eNOS genotypes (mean ± SD).

Parameter	GG (n = 129)	GT (n = 38)	TT (n = 19)	p-value
Nitrite (µmol∕ml)	16.35±2	16.22±3.6	18.1±4	P1 = 0.77 P2 = 0.002 P3 = 0.07
MDA (nmol∕ml)	2.21±0.2	2.1±0.4	2.33±0.4	P1 = 0.02 P2 = 0.03 P3 = 0.04

complications of diabetes. This model is based on the knowledge that increased intracellular glucose leads to the overproduction of super oxide by the mitochondrial electron transport chain and increased formation of secondary ROS. The activity of antioxidant systems is accordingly an important defense against ROS-associated cell injury (Brownlee, 2005). A unifying hypothesis has been proposed whereby mitochondrial production of ROS in response to chronic hyperglycemia may be the key initiator for these pathogenic pathways

(Evans et al, 2002). This increases the importance of mitochondrial dysfunction in the progression and development of diabetic complications including nephropathy. Despite positive preclinical research findings antioxidants per se have demonstrated minimal renal protection in humans with diabetes (Forbes et al., 2008). There are many sources of ROS in the diabetic kidney including autooxidation of glucose, advanced glycation, Xanthine Oxidase activity, peroxidases, NOS and NAD(P)H oxidase (Cross et al, 1987; Forbes et al, 2008). Elevated glucose concentrations may increase the levels of oxygen radical scavenging enzymes in cultured endothelial cells (Busik et al., 2008) and the kidney of rats with streptozotocin-induced diabetes (Koya et al., 1997). An imbalance between ROS generation and antioxidant capacity favoring the former leads to oxidative stress and oxidative damage.

The present study revealed no significant differences in the frequencies of genotypes and alleles between the studied diabetic patients with or without nephropathy when compared with control (p>0.05). These findings are in agreement with the results of Zanchi et al. (2000), who reported non-significant differences in the frequency of eNOS G894T genotypes in group of type 1 diabetics with nephropathy in comparison to another group of diabetics without nephropathy. However, the results of the present study disagree with those of Ahluwalia et al. (2008), who reported homozygous mutated TT genotype, and mutant T allele of G894T polymorphism with significant frequency among cases of types 1 and 2 diabetes with nephropathy compared to control and concluded that TT genotype and T allele may be considered genetic risk factors for diabetic nephropathy. Similarly, the study of Ezzidi et al. (2008) found that the frequencies of eNOS G894T gene genotypes and alleles in diabetics with nephropathy were significantly higher than those in diabetic patients without nephropathy. The discrepancy of the results between the different studies can be attributed to the variability of the number of patients studied or to the ethnic differences regarding the distribution of this pattern of polymorphism.

The present study also showed significant increase in the levels of NO and MDA as markers of oxidative stress in diabetic patients with or without nephropathy in comparison to control (all P<0.0001), also, there were significant increase in the levels of NO and MDA in diabetics with nephropathy when compared with diabetics without nephropathy (p = 0.02 and 0.001 respectively).There was significant increase in the level of NO in TT genotype when compared with GG genotype (p = 0.002), also, there was significant increase in the level of MDA in TT genotype when compared with GT ,GG genotype (p = 0.04, p = 0.03 respectively).

Increased amounts of eNOS have been found in pre-glomerular blood vessels in diabetic rat and NOS-inhibitors have been shown to prevent increase in glomerular filtration rate (Veelken et al., 2000). These findings suggest that NO and the NOS enzymes (particularly eNOS), can contribute to the glomerular damage leading to nephropathy in diabetic patients (Mollsten et al., 2009). No association was found between the serum NO levels and the Glu298Asp polymorphism in both control and patient groups. Many studies concerning the association between genetic polymorphisms of eNOS and serum NO concentrations have been previously reported. A study examined whether the intron 4 variable number tandem repeat (VNTR), −922A/G, −786T/C and G894T polymorphisms in the eNOS gene influence NO production and showed that none of the studied polymorphisms had influenced the serum NO even if corrected for the serum creatinine level (Jeerooburkhan et al., 2001). Another study demonstrated that the median serum NO level in G/T and T/T genotype of the G894T polymorphism was significantly higher than that of G/G genotype in the healthy population but not in the CAD patients (Yoon et al., 2000).

Conclusion

The present study revealed no significant differences in the frequencies of genotypes and alleles between the studied diabetic patients and the control group. The higher serum levels of markers of oxidative stress in diabetic patients with nephropathy suggest the possible role of oxidative stress but not endothelial Nitric Oxide Synthase gene polymorphism in pathogenesis of the disease. Further studies on a wider scale of diabetic patients suffering from either type 1 or type 2 at different progressive levels of the disease are needed.

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