Role of nitric oxide in the pathogenesis of renal hemodynamic changes in diabetes

Diyabette görülen renal hemodinamik değişikliklerin patogenizinde nitrik oksidin rolü

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This work was supported by the Research Foundation of Erciyes University.

This manuscript can be downloaded from the webpage:
http://tipdergisi.erciyes.edu.tr/download/2007;29(3)189-194.pdf

Submitted : April 20, 2006
Revised : May 17, 2007
Accepted : May 26, 2007

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Abstract
Background: Several reports suggest that increased generation or activity of nitric oxide (NO) have been implicated in the pathogenesis of glomerular hyperfiltration and hyperperfusion that occurs in early diabetes. However, the precise role of altered NO generation in the pathogenesis of diabetic nephropathy is unclear. The present study was aimed to investigate the role of NO in the pathogenesis of glomerular hyperfiltration and hyperperfusion in streptozotocin (STZ)-induced diabetic rats.
Materials and Methods: The renal hemodynamic and renal function parameters, such as glomerular filtration rate (GFR), renal blood flow (RBF), and creatinine clearance, protein, sodium and potassium levels were determined. The above mentioned parameters were measured before and after administration of a nonspecific NO synthesis inhibitor, nitro-L-arginine methyl ester (L-NAME), in diabetic and control rats. Plasma NO and malondialdehyde (MDA), the last product of lipid breakdown caused by oxidative stress, levels were also measured.
Results: Diabetic rats exhibited significantly elevated plasma NO levels, approximately two-fold higher than controls ($p<0.001$). L-NAME treatment prevented an increase in plasma NO concentrations in diabetic rats. GFR, RBF, and creatinine, protein, sodium and potassium excretions were significantly elevated in the diabetic animals. Inhibition of NO synthesis by L-NAME attenuated the RBF and GFR in diabetic rats but had no effect on the other parameters. L-NAME treated diabetic rats had a marked decrease in plasma MDA levels compared to the diabetes mellitus and control group.
Conclusion: Renal hyperfiltration and hyperperfusion in diabetic rats increased in correlation with raised plasma NO levels. When NO is blocked, renal hyperfiltration and hyperperfusion in diabetic rats decreased. This suggests that the increase of NO levels might not be a single factor that is responsible for the changes in the pathogenesis in early diabetes.

Key Words: **Blood pressure; Diabetes Mellitus; Glomerular Filtration Rate; Nitric oxide; Renal circulation.**

Özet
Giriş: Pekçok çalışmada, diyabetin erken döneminde nitrik oksit yapım ve etkisindeki artışın glomerüler hiperfiltrasyon ve hiperperfüzyon patogenezinden sorumlu olduğu bildirilmektedir. Fakat nitrik oksit yapımındaki değişikliğin, diyabetik nefropatinin patogenezindeki rolü tam olarak belirlenememiştir. Çalışmanın amacı, streptozotosin ile deneysel diyabet oluşturulan sıçanlarda glomerüler hiperfiltrasyon ve hiperperfüzyon patogenezinde nitrik oksitin rolünü açıklayabilmektir.
Gereç ve Yöntem:Çalışmamızda, diyabetik ve kontrol sıçanlarda, glomerüler filtrasyon hızı, renal kan akımı, kreatinin klirensi, protein, sodyum, potasyum atım düzeyleri gibi renal hemodinami ve renal fonksiyonları gösteren parametrelere bakılmıştır. Diyabetik ve kontrol sıçanlara non spesifik nitrik oksit sentez inhibitörü, nitro-L-arjinin metil ester (L-NAME) verilmeden ve verildikten sonra yukarıdaki parametreler değerlendirilmiştir. Gruplarda plazma nitrik oksit ve malondialdehit düzeyleri de belirlenmiştir.
Bulgular: Diyabetik sıçanlarda plazma nitrik oksit düzeyleri önemli derecede yükselmiştir. Kontrol değerlerinin yaklaşık iki misli düzeyde bulunmuştur. L-NAME verilmesi diyabetik sıçanlardaki artan nitrik oksit düzeylerini düşürmüştür. Glomerüler filtrasyon hızı, renal kan akımı, ve kreatinin, protein, sodyum, ve potasyum atılımları diyabetik hayvanlarda önemli derecede artmıştır. Diyabetik sıçanlarda, L-NAME ile nitrik oksit sentezinin azaltılması, renal kan akımı, ve glomerüler filtrasyon hızını azaltmış fakat diğer parametreler üzerine önemli etkisi bulunmamıştır. L-NAME verilen diyabetik sıçanlarda malondialdehit düzeyleri kontrole göre önemli derecede azalmıştır.
Tartışma: Diyabetik sıçanlarda renal hiperfiltrasyon ve hiperperfüzyon artmış plazma nitrik oksit düzeyleri ile ilişkili bir şekilde artmıştır. Nitrik oksit yapımı azaltıldığında diyabetik sıçanlardaki hiperfiltrasyon ve hiperperfüzyon azalmış ama tam olarak normale dönmemiştir. Diyabetin erken dönem patogenezinde gelişen olumsuz yöndeki değişimlerden tek sorumlu faktör diyabetle artmış nitrik oksit düzeyleri olmayabilir.

Anahtar Kelimeler: **Glomerüler Süzüntü Hızı; Kan basıncı ; Nitrik oksit; Renal dolaşım; Şekerli diyabet.**

Introduction

According to the recent Diabetes Complication and Control Trial, even with very tight glycemic control, up to 50% of patients may progress to nephropathy (1). The initial stages of clinical and experimental insulin dependent diabetes mellitus are characterized by pronounced glomerular hyperperfusion and hyperfiltration as demonstrated previously (2,3). Several mechanisms have been proposed to explain the intraglomerular hyperfiltration state. These include increased production of vasodilatory prostaglandins, impaired responsiveness to thromboxane, increased level of atrial natriuretic peptide, and chronic hyperglicemia (4). However, the exact pathogenetic mechanism of glomerular hyperfiltration remains unknown.

Endothelial dysfunction has been suggested as an early event in diabetic vascular disease and may be relevant to the pathogenesis of diabetic microangiopathy (5). Oxidative stress is believed to be enhanced in patients with diabetes mellitus, which may lead to endothelial dysfunction and development of atherosclerosis (5). Among the vasodilators of endothelial origin, the most important representative is nitric oxide. Nitric oxide might be one of the causes of glomerular hyperfiltration (4). It is produced from the conversion of L-arginine to L-citrulline by a family of enzymes, known as NO synthase (NOS). Veelken et al. reported that early glomerular hyperfiltration was dependent on increased NO generation due to greater expression and activity of endothelial NOS (eNOS) in glomeruli and afferent arterioles (6).

The role of NO in diabetes is still somewhat controversial, with studies demonstrating an increase (7-9), or decrease in NO synthesis (10,11) in diabetic state. The mechanisms by which changes in NO levels are related to alterations in renal hemodynamics have not been fully eluciated. In this model of early diabetic nephropathy, our aim was: (a) to examine the effects of early streptozotocin-induced diabetes on renal hemodynamics and function and (b) to assess whether NO plays a pivotal role in the development of glomerular hyperfiltration by preventing NO synthesis using a NO synthesis inhibitor.

Materials and Methods

Animals. All procedures were approved by the Ethics Committee of Erciyes University. Studies were conducted in adult male Wistar albino rats, initially weighting 291.5±30.6g. All animals were housed individually in standard rat cages in a room on a 12 hour light/dark cycle at 22ºC. Forty Wistar albino rats were divided into three groups, ensuring that the animals in each group had the same mean body weight: Control (C, n=10); diabetic (DM, n=15) and nitro-L-arginine methyl ester (L-NAME) given to diabetic rats (DM, L-NAME, n=15). L-NAME is a nonspecific NO synthesis inhibitor (Sigma Chemical Co, St Louis, MO, US). In each experimental group, the rats were injected intravenously with a single 60mg/kg dose of streptozotocin. L-NAME was administered by intraperitoneal injection during the last three days of the experiment (40 mg/kg). Blood glucose concentration was evaluated weekly by reflectometery (Model mediSense, Precision Q.I.D, USA) in blood samples obtained from the tip of the tail. Diabetic rats were kept moderately hyperglycemic (300-500mg/dl) by daily subcutaneous injections of 2-4 units of NPH insulin. On day 15, the rats were placed in metabolic cages for 24 hours to measure food and water consumption, urine output, and the electrolytes, creatinine and protein concentration of urine. Some animals in the diabetic groups were lost during the experiment: 4 from DM group and 6 from DM, L-NAME group.

Cardiovascular Measurement:

On day 15 , the animals were anesthetized with a mixture (0.4ml/Kg) of Fentanyl (50µg/ml, 5ml) and Midazolam (15µg/ml, 2ml) Polyethylene catheters (PE50) were inserted into the femoral artery to measure the mean arterial blood pressure (MAP), systolic pressure (SP) and diastolic pressure (DP). All the catheters were filled with heparinized normal saline. The body temperature was maintained at 37ºC. Two hours after anesthesia, MAP, SP and DP were recorded with a pressure transducer (BIOPAC systems Instrument, California). Renal blood flow was measured by using a Doppler flow probe (BIOPAC systems instruments, ISD-140). All the cardiovascular parameters were monitored on a polygraphic recorder interfaced to a computer system.

Analytical Techniques:

At the end of the experiment, the plasma was separated from the red blood cells by centrifuging the blood at 3000rpm for 5 minutes. Plasma aliquots were stored at -20ºC for determination of NO, MDA and creatinine. The plasma concentrations of creatinine were measured using an automated analytical system (Konelab 60i,Intelligent Diagnostic Systems, Espoo, Finland). Urine creatinine, protein, glucose, sodium (Na^+), potassium (K+) and

phosphorus (P) levels were measured using the same analytical system.

Malondialdehyde Masurement. Lipid peroxidation was evaluated by thiobarbituric acid reactive substances method (TBARS). This method evaluates the oxidative stress assayed for malondialdehyde, the last product of lipid breakdown caused by oxidative stress (12).

Nitric Oxide Measurement. The NO formation was measured by the spectrophotometric Griess method, modified by Arto and Sandra in 1996, which is based on the conversion of nitrates into nitrites in the presence of the nitrate reductase (13).

Calculations and Statistical Analyses. Data are given as means ± SD. All statistical analyses were performed by using Sigma Stad TM 10.0 (SPSS Inc., Chicago, IL, USA). The normality of distribution was checked. The data were normally disturbed, statistical evaluation was performed by using ANOVA test (Kruskal-Wallis). Statistical significances were accepted only when $p<0.05$.

Results

The initial body weights of the rats ranged between 217 and 343g. As expected, significant differences were seen between the groups in terms of body weight, water consumption, urine output and blood glucose after 2 weeks of STZ –induced diabetes (Table I).

Diabetes increased the plasma NO level (~103 %, $p<0.001$) compared to those of the control group. On the other hand, L-NAME treatment decreased the plasma NO level in the diabetic rats. MDA levels increased in the diabetes group compared to those of the control group (Table II). Diabetic rats exhibited an increase in RBF of 131% compared to the control group (C: 705.8±92.2, DM: 1634.9 ± 171.0, $p<0.003$) (Table III). L-NAME treatment in diabetic rats decreased RBF by 45% compared to diabetic rats ($p<0.003$). Diabetic rats showed an eight fold increase in urinary flow rate, and a modest increase in glomerular filtration rate and creatinine clearance compared to the control rats. The diabetic rats exhibited increases in creatinine excretion (DM: 53.8±32, C:9.39±4.96), protein excretion (DM: 0.75±0.64; C: 0.11±0.07), Na^+ excretion (DM: 99.7±58.6; C;5.35±3.7), K^+ excretion (DM:202.0±102.5; C:10.3±7.9) and P excretion (DM:144.52 ± 79.77; C:12.39±9.01). Although it did not reach a significant level, L-NAME treatment in diabetic rats caused a decrease in protein, Na^+, K^+ and P excretions.

STZ-induced diabetes caused slightly decreases in MAP, SP and DP. L-NAME treated diabetic rats exhibited a significant ($p<0.05$) increase in MAP, SP and DP, compared to the control and diabetic rats. STZ-induced diabetes caused a significant decrease in heart rate compared to the control group (C: 366.2±41.2, DM: 256.1± 30.0 $p<0.0001$) and when L-NAME was given to the diabetic rats, the heart rate became close to the control level (DM+L-NAME; 311.1±32.3, DM; 256.1±30.0, $p<0,001$).

Discussion

Dilatation of afferent glomerular arterioles is one of the characteristic changes in the early stage of diabetic nephropathy. Inappropriate dilatation of afferent arterioles is believed to induce glomerular hyperfiltration and hyperthrophy followed by thickening of the glomerular basement membrane and accumulation of mesengial matrix. eNOS is expressed in afferent arterioles and glomeruli (14,15). In this study, plasma NO levels were higher in the diabetic groups than controls. RBF and GFR increased in the diabetic rats compared to the controls. In accordance with the literature, the NO levels in the present study were found to be enhanced in diabetic animal and humans (4,16). GFR and RBF have shown that the renal microcirculation and mesengial expansion are correlated to the glomerular enlargement in the early stage of diabetic nephropathy (6,17). Several reports suggest an important role of NO in preferential afferent arteriolar dilatation, glomerular enlargement and functional glomerular hyperfiltration in the early stages of diabetic nephropathy (4). In this study, treatment with L-NAME, a nonselective NO inhibitor of NOS, attenuated hyperfiltration with no change in blood glucose level. It has been demonstrated that the prolonged exposure of endothelial cells to high glucose levels increase both NO and superoxide anion production (18). In the present study, increased plasma MDA levels reflect increased lipid peroxidation in the diabetic groups. Ha H. et al indicated that four weeks after STZ-induced diabetes, rats exhibited a 2.2 fold increase in urinary lipid peroxidation excretion compared with control rats (19). However, inhibition of NO production in the DM group decreased lipid peroxidation. Furthermore, Gonzales et al. suggested that the removal of oxygen species by incubating pancreatic tissues led to a decrease in nitrite levels (42%) and NO

synthase (NOS) activity (50%) in diabetic animals but not in control samples. When NO production was blocked by L-NAME, SOD (Superoxide dysmutase) activity increased (20). This suggests that inhibition of NOS activity produces an increase in SOD activity and a decrease in lipoperoxidation in diabetic pancreatic tissues. Ishii et al indicated that the early stage of diabetes mellitus provokes accelerated renal cortical superoxide anion production in a setting of normal or increased NO production (21). It is postulated that the increasing clearances of creatinine, protein, sodium and potassium in DM may be related to accelerating superoxide anion production in early stage of diabetes.

The findings of this study show that STZ-induced diabetes caused a modest decrease in arterial pressure (MAP, SP and DP) compared to control group after the two weeks of diabetes. In this study, diabetic rats were treated with daily insulin injections to avoid excessive hyperglycemia and/or ketosis, thus more closely approaching the metabolic conditions prevailing in diabetic patients. Some experimental studies have shown that the onset of diabetes in rats is associated with an increase or decrease in arterial pressure (8,22). The reason for the discrepancies in different studies is not known but could be due to the influence of ambient glucose concentrations at the time of the study, tissue specific responses, or differences in the duration of diabetes at the time of testing.

These results indicate that an ability to synthesize NO is important at the onset of diabetes in order to prevent hypertension, and perhaps to enable hyperfiltration and scavenge increased superoxide anion in DM. Therfore, we speculate that the renal vasodilator action of NO may be a mechanism that prevents an increase in arterial pressure during the early stages of diabetes. The increased NO levels may be partly responsible from the glomerular hyperfiltration and hyperperfusion in the DM and these effects of NO may be due to its marked increase in RBF. However, this is not the only factor that plays a role in this pathogenesis since in the L-NAME treated diabetic rats the RBF was still considerably much higher compared to the control group. The pathogenesis of the early stages of DM can also be associated with the increased oxidative stress, due to the fact that elevated superoxide and increased NO may play an important role in scavenging these superoxide anions in order to lessen the oxidative stress.

Acknowledgment. We thank to Dr.Yunus Dursun and Dr. Cem Süer for their help in the statistical and English manuscript, respectively.

Table I. Body weight, blood glucose level, water consumption and urine output in diabetic and control rats on day 15,with or without nitric oxide inhibition.

Parameters	**C(n=10)**	**DM (n=10)**	**DM,L-NAME (n=9)**	**F**	**P**
Body weight (g)					
On day 0.	284.7±46.0	298.2±22.6	294.8±16.4	00.49	0.616
On day 15.	290.8±26.1	241.2±26.6 [a]	256.0±12.6 [b,c]	12.17	0.000
Water Consumption (ml/day)	20.9±8.3	106.3±46.5 [a]	99.1±34.7 [a]	12.17	0.000
Urine (ml/day)	11.4±3.5	91.6±21.7 [a]	79.8±28.0 [a,b]	10.41	0.000
Blood glucose (g/dl)	165.2±26.6	391.8±56.7 [a]	417.5±72.0 [a,b]	24.76	0.000

Values are means±SD. **C;** control, **DM;** diabetes mellitus, **DM,L-NAME;** diabetes mellitus plus L-NAME.
a: Significantly different from C ($p<0.05$)
b: Significantly different from C ($p<0.05$)
c: Significantly different from DM ($p<0.05$)

Table II. Nitric oxide and lipid peroxidation product, malondialdehyde in diabetic and control rats on day 15, with or without nitric oxide inhibition.

Parameters	C (n=10)	DM (n=10)	DM,L-NAME(n=9)	F	P
NO (nmol/ml)	46.9±08.76	114.00±24.12 [a]	62.11±15.75 [b]	40.42	0.000
MDA(nmol/ml)	0.12±0.07	0.32±0.18[a]	0.27±0.04[bc]	7.40	0.003

Values are means±SD. **C;** control, **DM;** diabetes mellitus, **DM,L-NAME;** diabetes mellitus plus L-NAME.
NO; Nitric oxide, **MDA;** Malondialdehyde.
a: Significantly different from C (p<0.05)
b: Significantly different from DM (p<0.05)
c: Significantly different from C (p<0.05)

Table III. Renal blood flow (RBF), urinar output rate, creatinine clearence and urinary excretion of creatinine , protein, glucose, potassium, sodium and phosphorus excretions in diabetic and control rats on day 15,with or without nitric oxide inhibition.

Parameters	C (n=10)	DM (n=10)	DM,L-NAME (n=9)	F	P
RBF (bpu)	705.84±92.21	1634.94±171.02[b]	896.22±144.84 [a]	122.48	0.000
Urinary output (mg/min)	7.95±2.45	63.66±15.10 [b]	55.47±19.48 [a]	191.57	0.000
Creatinine clearance (ml/ min)	1.49±0.62	1.70±0.44	0.98±0.47	2.96	0.06
Creatinine excretion (mg/ min)	9.39±4.96	53.84±32.51 [b]	55.44±37.09 [a]	16.12	0.000
Protein excretion (mg/ min)	0.11±0.07	0.75±0.64	1.01±0.08 [a]	5.75	0.008
Glucose excretion (mg/ min)	0.0023±0.00	121.99±146.70 [b]	94.14±49.52 [a]	26.00	0.000
Sodium excretion (mol/ min)	5.35±3.76	99.71±58.65 [b]	114.29±83.60 [a]	50.12	0.000
Potassium excretion (mol/ min)	10.33±7.94	202.04102.55 [b]	174.5533.15 [a]	9.27	0.001
Phosphorus excretion (mol/ min)	12.39±9.01	144.5279.77 [b]	104.8965.57 [a]	12.96	0.000

Values are means±SD. **C;** control, **DM;** diabetes mellitus, **DM,L-NAME;** diabetes mellitus plus L-NAME.
RBF; Renal blood flow, bpu;blood per unit
a: Significantly different from C (p<0.003)
b: Significantly different from C (p<0.0001)

Table IV. Mean arterial, systolic, diastolic arterial pressure levels in diabetic and control rats on day 15,with or without nitric oxide inhibition

Parameters	C (n=10)	DM (n=10)	DM,L-NAME (n=9)	F	P
SP (mmHg)	107.85±19.33	101.78±14.54	128.18±15.55 [a,b]	9.62	0.001
DP (mmHg)	82.21±16.93	75.49±10.04	104.67±15.66 [a,b]	6.49	0.005
MABP (mmHg)	93.42±17.86	87.70±11.82	116.60±14.90 [a,b]	10.41	0.000
Heart rate (beat/min)	366.20±41.20	256.10±30.07[c]	311.11±32.38 [a,b]	11.46	0.000

Values are means±SD. **C;** control, **DM;** diabetes mellitus, **DM,L-NAME;** diabetes mellitus plus L-NAME.
SP; Systolic pressure, **DP;** Diastolic pressure, **MABP;** Mean arterial blood pressure,
a: Significantly different from C (p<0.05)
b: Significantly different from DM (p<0.05)
c: Significantly different from C (p<0.05)

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