# Inducible nitric oxide synthase (iNOS) expression in early diabetic nephropathy: Effect of ACE inhibitors and angiotensin II receptor antagonists

## *Erken diabetik nefropatide indüklenebilir nitrik oksid sentetaz (iNOS) ekspresyonu: ACE inhibitörlerinin ve anjiotensin II reseptör antagonistlerinin etkileri*

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***Summary***

***Objective:*** *One of the most important complications of diabetes mellitus is diabetic nephropathy. Captopril is an ACE inhibitor and used in the diabetic nephropathy treatment. In the last years, it has been shown that angiotensin receptor antagonists have similar efficiency as ACE inhibitors for prevention of proteinuria. The angiotensin II-$AT_1$ receptor antagonist Losartan is a new antihypertansive material that has been worked on this field. Nitric oxide (NO) is a multifunctional mediator that has been implicated in the short-term hemodynamic alterations that occur acute streptozocin induced diabetes. Nitric oxide is synthesized exclusively from its precursor L-arginine under the catalytic effect of nitric oxide synthase (NOS), which there are three isoforms. Diabetic nephropathy represents a complex metabolic arena characterized with patho-physiological events that both stimulate end depress intrarenal NO production. The aim of this study was to assess whether the iNOS pathway is pathologically altered in experimental diabetic nephropathy, and in therapy with ACE inhibitor (Captopril) or angiotensin II-$AT_1$ receptor antagonist (Losartan).*

***Özet***

***Amaç:*** *Diabetes mellitusun en önemli komplikasyonlarından biri diabetik nefropatidir. Kaptopril bir ACE inhibitörüdür ve diabetik nefropati tedavisinde kullanılır. Son yıllarda anjiotensin reseptör antagonistlerinin proteinüriyi önlemek için ACE inhibitörleri gibi etki ettikleri gösterilmiştir. Anjiotensin II-AT1 reseptör antagonisti Losartan, bu çalışma alanında çalışılan yeni bir antihipertansif materyaldir. Nitrik oksit (NO), akut streptozosin verilerek oluşturulan diabetteki, akut meydana gelen hemodinamik değişikliklerde etkisi bulunan çok fonksiyonlu bir mediatördür. Nitrik oksit, nitrik oksit sentez (NOS) enziminin katalitik etkisi altında prekürsör L-arjinin'den sentezlenir ve 3 alt grubu vardır. Diabetik nefropati de böbrek içi NO üretimini hem artıran hem de azaltan patofizyolojik olaylarla karakterize karmaşık metabolik olayları içerir. Bu çalışmanın amacı, deneysel diabetik nefropatide iNOS yolunun patolojik değişiklikleri ve ACE inhibitörleri ve anjiotensin II-AT1 reseptör antagonisti kullananlardaki etkileridir.*

**Materials and methods:** 32 male Wistar rats weighting 140-220 g were used for this study. The rats were made diabetic using a single intraperitoneal injection of 65 mg/kg streptozotocin (Sigma Chemical Co., St. Louis, MO, USA). Rats were divided in to four groups: Group A (n= 8): Control, Group B (n=8): STZ- diabetic control, Group C (n=8): STZ + Losartan (DM+ L) (10 mg/kg/day), Group D (n=8): STZ + Captopril (DM+C) (50 mg/kg/day). The experiment lasted 6 weeks. The levels of blood glucose, albuminuria, creatinine clearence was determined. Renal tissue samples were extracted under anesthesia for histopathologic study. Monoclonal antibodie against the following antigen was used: iNOS (Zymed 61-7700).

**Result:** At the diabetic control group, the level of albuminuria was increased significantly and was decreased significantly in the Captopril and Losartan given groups ($p<0.001$). There was no statistically significant differences in albuminuria between the DM+L and DM+C groups ($p>0.05$). The values of creatinine clearence were increased in diabetic control group as compared to control group ($p<0.001$). The reducing creatinine clearence in DM+L and DM+C groups was statistically not significant ($p>0.05$). By immunohistochemistry, iNOS staining was only observed in glomeruli of diabetic control group.

**Conclusion:** Glomerular iNOS expression was enhanced in diabetic nephropathy and the activation of angiotensin II may play a role in this enhancement.

**Key words:** iNOS, diabetic nephropathy, ACE inhibitor, Angiotensin II

***Materyal ve metod:*** *Bu çalışmada 140-220 gr ağırlığında 32 adet Wistat ratları kullanılmıştır. Ratlarda tek doz intraperitoneal 65 mg/kg streptozotocin (Sigma Chemical Co., St. Loui, MO, USA) kullanılarak diabet oluşturulmuştur. Ratlar 4 gruba bölünmüştür: grup A (n=8): Kontrol, grup B (n=8): STZ-diabetik kontrol, grup C (n=8): STZ+Losartan (DM+L) (10 mg/kg/gün), grup D (n=8): STZ+Kaptopril (DM+C) (50 mg/kg/gün). Deney 6 hafta sürdürüldü. Kan şekeri düzeyi, albüminüri, kreatinin kleransı saptandı. Böbrek dokusu örnekleri histopatolojik çalışma için anestezi altında alındı. Monoklonal antikora karşı antijen kullanıldı: iNOS (Zymed 61-7700).*

***Sonuçlar:*** *Diabetik kontrol grubunda albiminüri seviyesi anlamlı derecede artmış ve Kaptopril ve Losartan verilen gruplarda önemli derecede azalmıştı ($p<0.001$). DM+L ve DM+C grupları arasında anlamlı bir istatiksel fark görülmemişti ($p>0.05$). Kreatinin kleransinin değeri kontrol grubuna benzer şekilde diabetik kontrol grubunda da artmıştı ($p<0.001$). DM+L ve DM+C gruplarında kreatinin kleransinindeki azalma istatiksel olarak anlamlı değildi ($p>0.05$). İmmün dokukimyasal olarak iNOS boyanması diabetik kontrol grubunda sadece glomerüllerde görüldü.*

***Tartışma:*** *Glomerüler iNOS ekspresyonu diabetik nefropatiyi arttırır ve anjiotensin II aktivasyonu bu artmada rol oynayabilir.*

***Anahtar sözcükler:*** *iNOS, diabetik nefropati, ACE inhibitörleri, Anjiotensin II*

Diabetic nephropathy is the most common single cause of end-stage renal disease in the United States and in Europe (1). Diabetic nephropathy is a long-term complication that occurs in 30-40% of patients with diabetes mellitus (DM) (2). Despite numerous studies, the pathophysiology of DM nephropathy is not completely understood. Increases of renal perfusion and GFR occur early in the course of diabetic nephropathy, a feature seen in experimental and clinical diabetes. Micropuncture studies demonstrated renal vasodilation predominantly of the preglomerular or afferent resistance vessels as a cause of diabetic hyperfiltration (1). This was reported to be due to increased formation of nitric oxide (NO) (3). NO was first shown to be indentical with endothelial derived relaxing factor in 1987 (4) and this was followed by a rapid flurry of information defining the significance of NO in not only vascular physiology and hemodynamics but also in neurotransmission, inflammation and immune defense systems. NO acts as a vascular and neural messenger activating soluble guanylate cyclase, resulting in increased levels of cGMP. NO originates from L-arginine in a reaction catalyzed by se-veral different nitric oxide synthase (NOS) isoenzymes. The family of NOS proteins are classified into: the constitutive type, neuronal NOS (nNOS), endothelial NOS (eNOS), and the inducible type (iNOS). iNOS can be expressed by various cell types, including macro-phages, vascular smooth muscle cells, and glomerular mesangial cells, leading to the formation of large amounts of NO and can be induced by endotoxins and cytokines (5). It has been proposed that stimulation of iNOS due to hyperglycemia may lead to increased generation of NO, which in turn contributes to diabetic hyperfiltration and glomerular abnormalities in diabetes (6).

Recent large-scale clinical studies have demonstrated that poor glycemic control, activation of the renin-angiotensin axis, and hypertension play an important role in the progression of diabetic nephropathy (7). Several autocrine and vasoactive factors including

angiotensin, eicosonoids, endothelin, kinins, atrial natriuretic peptide, transforming growth factor-β thromboxane, bradykinin and nitric oxide have been implicated in modifying the rate of progression of diabetic renal disease. NO could potentially play a major role in mediating the effects of hyperglycemia, hypertension, and activation of angiotensin (3, 8-10).

This study aimed the hypothesis that NO-mediated renal vasodilation due to the activity of the iNOS contributes to glomerular hyperfiltration in diabetic rats. We investigated the role of NO produced by iNOS in early STZ diabetic nephropathy, if therapy with ACE inhibitor (Captopril) or angiotensin II-$AT_1$ receptor antagonist (Losartan).

## Materials and methods

**Animals and experimental protocol:** Approval for the study was granted by the Medical Surgical Research Center of Osmangazi University and the Committee on Animal Experiments of the Medical Faculty of Osmangazi University. All experimental procedures were performed in accordance with the National Institute of Health's Principles of Laboratory Animal Care.

32 male Wistar rats weighting 140-220 g were used for this study. Animals were kept under standart laboratory conditions and allowed free access to food and water. The rats were made diabetic using a single intraperitoneal injection of 65 mg/kg streptozotocin (Sigma Chemical Co., St. Louis, MO, USA). 48 h later, blood glucose levels were determined using tail blood samples. Only rats with blood glucose levels >250 mg/dl were included.

Rats were divided in to four groups: Group A (n= 8): Control. Group B (n=8): STZ- diabetic control. Group C (n=8): STZ + Losartan (DM+ L) (10 mg/kg/day). Group D (n=8): STZ + Captopril (DM+C) (50 mg/kg/day). The experiment lasted 6 weeks. The levels of blood glucose, albuminuria, creatinine clearence was determined.

Morphological examination: Renal tissue samples were extracted under anesthesia for histopathologic study. Fragments of the renal were fixed for 24 hours in buffered 10 % formalin solution and the embedded in parafin, sectioned at 4 μm; the sections were stained with hematoxyline and eosin, coded, and examined by a pathologist (KB), who was unaware of the treatment received and sacrifece time. Mesangial hypercellularity was defined as mesangial regions that contained more than three cells.

**Immunohistochemistry:** Immunohistochemical investigation was performed in serial 4-μm sections mounted on poly-ι-lysine coated slides. Monoclonal antibody against the following antigen was used: iNOS (Zymed 61-7700). After microwave antigen retrieval, the sections were incubated with the antibodies overnight at 4°C. Immunostain visualization was achieved with the standard streptavidin-biotin peroxidase technique. The slides were stained with 3,3'-diamino-benzidine, counterstained with hematoxylin, and mounted. As a positive control, sections with pyelonephritis were used. The pathologist (KB) who evaluated the immunohistochemical specimens was blinded to the strain of individual animals.

**Statistical analysis:** Calculations were performed with SSPS 10.0. Differences among groups were evaluated using Tukey HSD tests. Results are presented as the mean± SEM. Findings were considered significant if the *p* value was less than 0.05.

## Results

**Laboratory findings:** In our study, at the end of the second days and six weeks after administration of STZ, blood glucose levels was increased significantly in diabetic control, DM+L, DM+C groups as compared to control group ($p<0.001$). At the diabetic control group, the level of albuminuria was increased significantly and was decreased significantly in the Captopril and Losartan given groups ($p<0.001$). There was no statistically significant differences in albuminuria between the DM+L and DM+C groups ($p>0.05$). The values of creatinine clearence were increased in diabetic control group as compared to control group ($p<0.001$). The reducing creatinine clearence in DM+L and DM+C groups was not statistically significant ($p>0.05$). The laboratory findings are showen in Table I.

No significant gross pathology was recorded in any of the rats at autopsy. In glomeruli isolated from DM+C, DM+L and control rats no iNOS expression was detected. In contrast, positive staining were found in glomeruli of diabetic rats.

**Histology and ımmunohistochemical staining for iNOS:** Cytoplasmic clear cell change of the distal tubular epithelium (Fig 1), glomeruler basal membran thicking (Fig 2), glomeruler hypertrophy, mesangial cell increase (Fig 3) and tubuler proteinous cast were present in the diabetic control rats. Fibrinoid necrosis of glomeruler tuft

Table I. Blood glucose, albuminuria, and creatinine clearence in groups

| Parameter | | Control | DM | DM+L | DM+C |
|---|---|---|---|---|---|
| Blood glucose (mg/dl) | Week 0 | 112±6.6 | 251±18.7 | 250 ±23.3 | 240±21.9 |
| | Week 6 | 120±9.4 | 355±16.6* | 347±32.7* | 351±22.4* |
| Albuminuria (mg/dl) | | 220±48 | 826±53* | 490±84* | 443±72* |
| Creatinine clearence (mg/dl) | | 1.52±0.2 | 2.94±0.4* | 2.67±0.2** | 2.70±0.3** |

Data are expressed as mean ± SEM. C: Captopril L: Lasortan,
*p<0.001 **p>0.05

Table II. Histological findings and immunohistochemistry staining features for iNOS in groups

| | Control | Diabetic Control | DM+ Captopril | DM+ Lasortan |
|---|---|---|---|---|
| Glomerular basal membrane thickening | - | 8 | 1 | 2 |
| Glomeruler hypertrophy | - | 8 | - | - |
| Mesangial hypercellularity | - | 8 | 2 | 4 |
| Fibrinoid necrosis of glomeruler tuft | - | 5 | - | - |
| Tubuler proteinous cast | - | 8 | - | - |
| Cytoplasmic clear cell change of the distal tubular epithelium | - | 8 | 2 | 2 |
| İNOS staining | - | 8 | 2 | 1 |

(Fig 3) was present in five of the six diabetic control rats.

Glomeruler basal membrane thickening was present in one of the six Captopril treated rats and in two of the six Losartan treated rats. Mesangial cell increase was observed in two of Captopril treated rats and in four six Losartan treated rats. Cytoplasmic clear cell change of the distal tubular epithelium was present in two of Captopril treated rats and Losartan treated rats.

In diabetic control group rats, the glomerular mesangium were positive for iNOS in six rats (Fig 4). There was positive staining for iNOS within the glomeruli of two Captopril treated rats and one Losartan treated rat. No control rats showed positivity with iNOS. Histological findings and immunohistochemical staining for iNOS in groups were showed in Table II.

## Discussion

Recent large-scale clinical studies have demonstrated that poor glycemic control, activation of the renin-angiotensin axis, and hypertension play an important role in the progression of diabetic nephropathy (7). Several autocrine and vasoactive factors including angiotensin, eicosonoids, endothelin, and NO have been implicated in modifying the rate of progression of diabetic renal disease. NO could potentially play a major role in mediating the effects of hyperglycemia, hypertension, and activation of angiotensin (11). In the kidney, NO controls both afferent and efferent vascular tone, the ultrafiltration coefficient, and medullary blood flow (12). Some findings support a role for excessive NO production in mediating increased intraglomerular perfusion and pressure during the early stages of diabetes (13) as our study findings. In contrast, there is evidence that decreased production or inactivation of NO in long-term diabetes and alterations in vascular reactivity and tone contribute to the development of nephropathy (14). Thus, administration of 1% L-arginine-supplemented drinking water for 14 weeks to rats with STZ diabetes ameliorated proteinuria and diminished the extent of glomerulosclerosis. Several difficulties hinder investigations into the role of NO in diabetic nephropathy. First, NO exerts, many biological functions within a single organ system, including the kidney, making it difficult to make a definitive statement about the beneficial or deleterious effect of NO in a specific condition (11). Secondly, all three NOS isoforms are present in the kidney and play distinctive roles in the regulation of glomerular and tubular function (15). Third, agents that safely and selectively inhibit the activity of one isoform are not available for long-term studies. Therefore, at presents one feasible approach to assess the role of one NOS isoform in the pathogenesis of

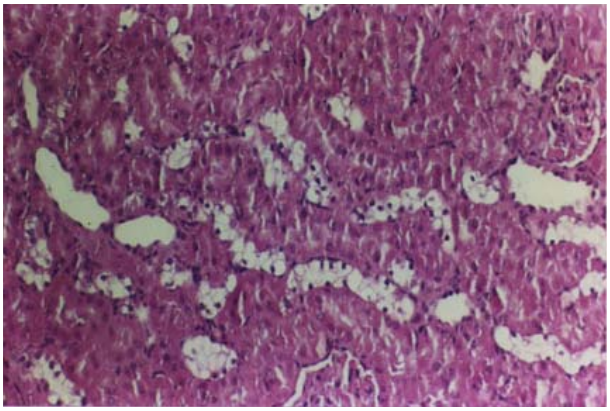

Fig 1. Cytoplasmic clear cell change of the distal tubular epithelium (H+E X40).

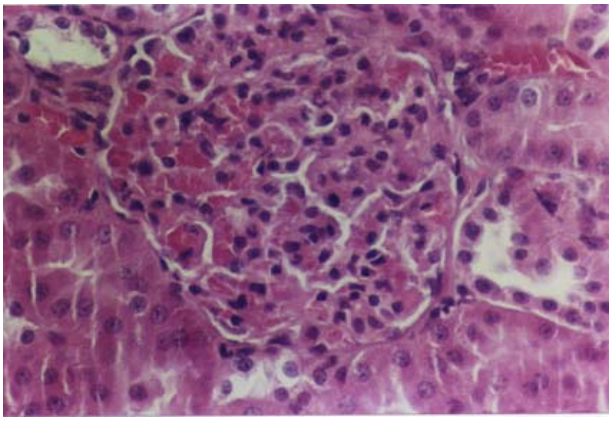

Fig 3. Glomeruler hypertrophy, mesangial hypercellularity and fibrinoid necrosis of glomeruler tuft (H+E X200).

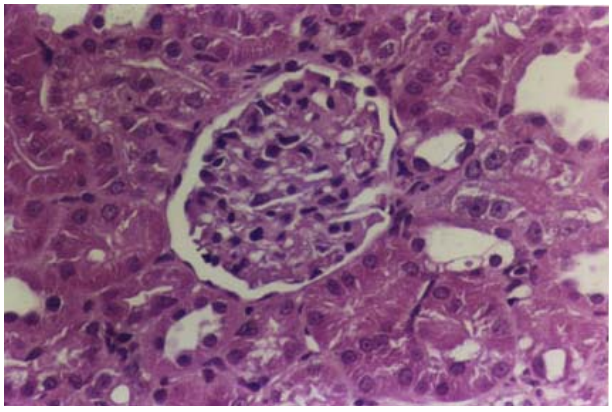

Fig 2. Glomeruler basal membrane thickening (H+E X100).

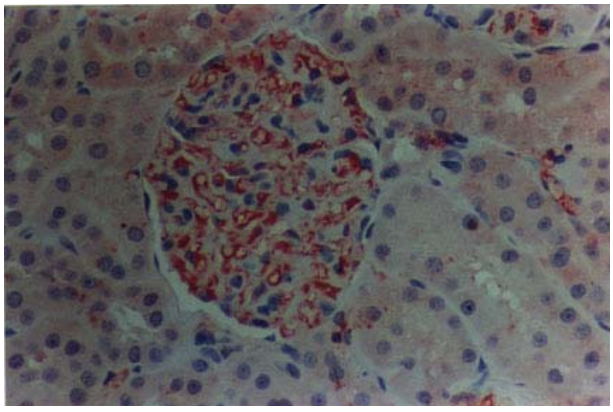

Fig 4. Immunohistochemical staining for iNOS is positive in glomerular mesangium of diabetic rats (iNOS X200).

disease is to use strains of mice with targeted genetic deletion of one form of the enzyme.

Acute inhibition of NOS with a low dose of the nonselective NOS inhibitor (L-NAME) decreased GFR and RPF significantly in diabetic animals back to normal control values, whereas this dose did not affect renal hemodynamics in control rats. This finding indicates that glomerular hyperfiltration is markedly dependent on increased NO generation and action at this early stage of experimental diabetic nephropathy. These results agree with previous reports, which postulated a significant role of NO in diabetic hyperfiltration (16), even though the involvement of specific NOS isoforms in this process remains unclear.

Since there is enhanced production of a variety of cytokines including TNF-α and IFN-γ in diabetes and macrophages infiltrated into the glomeruli of rats in the earliest stages of diabetes, it can be postulated that the cytokine-induced iNOS pathway may play an important role in glomeruler injury seen in diabetic kidneys. The exact role of iNOS in mediating DM nephropathy has not been fully elucidated, and the results of previous studies have been inconsistent. Some authors have determined an inhibition of induced NO production in cultered rat MCs by certain proinflammatory stimuli (17). Others were able to demonstrate an augmented LPS- or cytokine-induced NO production in murine MCs (7). Noh and et al. (18) reported that exposure of rat MCs to high glucose led to modest, but significant increases in iNOS

mRNA, protein expression and NO production. Lee and et al. (19) showed that LPS-stimulated iNOS expression is increased in diabetic glomeruli at the levels of mRNA and protein. Sugimoto et al. (20) reported that the expression of iNOS significantly increased in the rat glomeruli at 52 weeks after the induction of diabetes. They suggested the sequential pathway of advanced glycation end-products-cytokine-iNOS in the development of diabetic nephropathy.

Angiotensin II leads to renal vasoconstriction, regulating glomeruler perfusion, as well as filtration, and directly influences the mesangial constrictive response. Angiotensin II and NO have been postulated to interact closely. Yates et al. have recently show that angiotensin II inhibited iNOS production from cytokine stimulated mesangial cells and this effect was associated with increased transforming growth factor-β expression showed by immunocytochemistry (21). Conflicting data has been published regarding the effect of angiotensin II on iNOS expression in various experimental models. Mehta et al. (22) reported that the manipulation of the renin-angiotensin system, with antisense-oligodeoxy nucleotides directed at ACE mRNA, inhibits the expression of the iNOS in the myocardial ischemia model.

Clinically, the use of ACE inhibitors in diabetic nephropathy patients is well established (8, 23, 24). The effects of ACE inhibitors in diabetic nephropathy are due not only to hemodynamics but also enhanced NO action via suppressed bradykinin breakdown by ACE. Angiotensin II stimulates superoxide production via enhancement of NAD(P)H oxidase (25, 26), thus ACE inhibitor may have an effects to reduce superoxide production. Even though it has various beneficial effects, ACE inhibitor alone is not enough to normalize the proteinuria and to stop the progression of renal damage completely.

Schwobel et al. (27) demonstrated that induced NO production is negatively controlled by the angiotensin II type 2 receptor, whereas AT1 stimulation enhanced NO synthesis in MCs. Our data shows that the up-regulation of iNOS expression was ameliorated by ACE inhibitor or AT1 blocker, suggesting angiotensin II mediates the enhancement of iNOS expression via AT1 in diabetic glomerular injury.

Veelken et al. (1) found no functional or molecular evidence for increased glomerular expression and activity of iNOS in diabetes mellitus. In a recent study, iNOS and ecNOS protein expression was reported to be increased in kidneys of rats with STZ-induced diabetes mellitus (28). These investigations, however, were limited to Western blot analyses of total renal cortex and no data were provided on isolated glomeruli. Furthermore, it is unclear whether the observed increases of iNOS protein expression was effected by nonsterile surgery in anesthetized rats.

Our study showed the alteration of the iNOS pathway in diabetic glomerulopathy and the contribution of angiotensin II in the changes of the iNOS system. These results may implicate the iNOS pathway as a potential mediator of DM nephropathy. We confirmed that 6 wk of STZ diabetes led to glomeruler basal membrane thickening, glomerular hypertrophy and increased albuminuria.

In conclusion, the present study results confirm that NO plays an important role for the development of glomerular hyperfiltration in early diabetic nephropathy of rats. It has been shown that Captopril and Losartan has identical potence at prevention of albuminuria. Also, it has been shown that Captopril and Losartan have no effect on increased creatinine clearence.

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