Yonsei Medical Journal
Vol. 43, No. 2, pp. 183 - 192, 2002

Original Article

Inducible Nitric Oxide Synthase (iNOS) Expression Is Increased in Lipopolysaccharide (LPS)-Stimulated Diabetic Rat Glomeruli: Effect of ACE Inhibitor and Angiotensin II Receptor Blocker

Ho Yung Lee[1,2], Hyunjin Noh[1], Jingu Gang[1], Zhong Gao Xu[1], Hyeon Joo Jeong[1,3], Shin Wook Kang[1,2], Kyu Hun Choi[1,2], and Dae Suk Han[1,2]

[1]Institute of Kidney Disease, Departments of [2]Internal Medicine and [3]Pathology, Yonsei University College of Medicine, Seoul, Korea.

Previously, we reported that high glucose enhanced cytokine-induced nitric oxide (NO) production by rat mesangial cells (MCs), and that the enhanced expression of the iNOS pathway may promote extracellular matrix accumulation by MCs. The present study was designed to examine whether the iNOS pathway is pathologically altered in experimental diabetic nephropathy, and whether therapy with angiotensin converting enzyme (ACE) inhibitor (imidapril: I) or angiotensin II type I receptor (AT1) blocker (L-158,809: L), ameliorates these changes. Male Sprague-Dawley rats were injected with diluent (control: C) or streptozotocin. At sacrifice after 4, 8 and 12 weeks, rats underwent either a 4 hour placebo or an intraperitoneal lipopolysaccharide (LPS, 2 mg/kg) challenge. Systolic blood pressure (SBP) and urinary protein excretion (UPE) increased significantly in diabetic (D) rats compared with C. The basal expression of glomerular iNOS mRNA was increased in D rats compared with that of C rats, by reverse-transcription (RT)-polymerase chain reaction (PCR), whereas there was no significant difference in the level of protein by Western blot analysis. Upon LPS stimulation, the iNOS mRNA and protein expression was significantly elevated in D rats. In D rats, this up-regulation, of LPS-stimulated iNOS expression, was equally ameliorated both by I and L in mRNA and protein levels. From immunohistochemistry (IHC), there was a negative staining for the iNOS within the glomeruli of five C rats without LPS treatment, but one of four rats, with LPS treatment, showed minimal iNOS staining in the glomeruli. In D rats, the glomerular mesangium and podocytes were positive for iNOS in each of three out of five rats with, and without, LPS treatment.

In conclusion, LPS-stimulated 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, angiotensin II

Received August 24, 2001
Accepted January 10, 2002

This study was supported in part by a faculty research grant of Yonsei University College of Medicine for 2000 (No. 2000-05) for Dr. H.Y. Lee.

Reprint address: requests to Dr. Ho Yung Lee, Division of Nephrology, Department of Internal Medicine, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea. Tel: 82-2-361-5423, Fax: 82-2-364-7655, E-mail: hyl@yumc.yonsei.ac.kr

INTRODUCTION

Diabetic nephropathy is the leading cause of end-stage renal disease and one of the serious microvascular complications in diabetes mellitus (DM). Despite numerous studies, the pathophysiology of DM nephropathy is not completely understood. One of the mediators implicated in the development of DM nephropathy is the NO system.[1] NO acts as a vascular and neuronal messenger activating soluble guanylate cyclase, resulting in increased levels of cGMP. The family of NOS proteins are classified into; the constitutive type, neuronal NOS (nNOS), endothelial NOS (eNOS), and the inducible type. Within the kidney, high levels of nNOS are located in the macula densa. eNOS is found in glomerular afferent and efferent arterioles, with iNOS being localized in the cells of the mesangium.[2] Since there is enhanced production of a variety of

cytokines including TNF-α and IFN-γ in diabetes[3] and macrophages infiltrated into the glomeruli of rats in the earliest stages of diabetes,[4] it can be postulated that the cytokine-induced iNOS pathway may play an important role in glomerular injury seen in diabetic kidneys. However, 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 reported an inhibition of induced NO production in cultured rat MCs by certain proinflammatory stimuli.[5] Others were able to demonstrate an augmented LPS- or cytokine-induced NO production in murine MCs.[6] In our own study, we reported that exposure of rat MCs to high glucose led to modest, but significant increases in iNOS mRNA, protein expression and NO production.[7]

The aim of this study was to assess whether the iNOS pathway is pathologically altered in experimental diabetic nephropathy, and if therapy with ACE inhibitor or AT1 blocker ameliorates these changes.

MATERIALS AND METHODS

Animals and experimental protocol

All animal experiments described in this article were conducted in accordance with the Yonsei University Medical College guide for the care and use of laboratory animals. Male Sprague-Dawley rats, weighing 250 to 300 g, were made diabetic using a single intraperitoneal injection of 65 mg/kg streptozotocin (Sigma Chemical Co., St. Louis, MO, USA). Twenty-four hours later, blood glucose levels were determined using tail blood samples. The rats were housed in a temperature-controlled room, were given free access to water and standard laboratory chow during the study period.

Every 4 weeks, body weight (BW), SBP, and UPE were measured. At week 4, 8 and 12, the rats received either a 4 hour placebo, or an intraperitoneal LPS (2 mg/kg, Sigma Chemical Co.) challenge, and were then sacrificed. Kidney weights were measured at the time of sacrifice. Coronal slices of kidney, for routine light microscopy, were processed in the standard fashion, and the kidney remnants were prepared for the isolation of glomeruli. Glomeruli were prepared using a sieving technique as previously described,[8] and the samples stored at -70℃ in preparation for the analytical procedures described later in this article. Thin kidney slices, from the rats sacrificed at 12 weeks, were processed for IHC studies.

The effect of ACE inhibitor, or AT1 blocker, on the expression of glomerular iNOS was also examined in LPS-stimulated D rats. Diabetes was induced by the same method as above, and all the rats received LPS stimulation at sacrifice. Four groups were studied for 12 weeks. The first (n=6) and second groups (n=7), were C and D rats without drug treatment respectively; the third group (n=7) were D rats treated with ACE inhibitor (I, 2 mg/kg/day, Donga Pharmaceutical); and the fourth group (n=7) were D rats treated with AT1 blocker (L, 1 mg/kg/day, Merck Sharp and Dohme). I or L was given by gavage. Every 4 weeks, BW, SBP, and UPE were measured. At week 12, the expression of the iNOS mRNA and protein was examined.

Analytical methods

Blood glucose was measured by a glucometer, and 24 hour UPE was determined by the Bradford method, using the Bio-Rad assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). SBP was measured by the tail-cuff method.

Total RNA extraction

Total RNA was extracted by the addition of 100 μL of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA) to the glomeruli, followed by glomerular lysis by freezing and thawing three times. Another 700 μL of RNA STAT-60 reagent was added, and the mixture was vortexed, and stored for five minutes at room temperature. Four hundred microliters of chloroform were added, and the mixture shaken vigorously for 30 seconds. After three minutes, the mixture was centrifuged at 12,000 $\times$ g for 15 minutes at 4℃. The upper aqueous phase, containing the extracted RNA, was transferred to a new tube. RNA was precipitated from the aqueous phase by 400 μL isopro-

panol, and pelleted by centrifugation at 12,000 × g for 30 minutes at 4℃. The RNA precipitate was washed with 70% ice-cold ethanol, dried using Speed Vac, and dissolved in DEPC-treated distilled water. The RNA yield and quality were assessed based on spectrophotometric measurements at 260 and 280 nm for OD.

RT-PCR

First-strand cDNA was made using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Total RNA from glomeruli was reverse transcribed using 10 μmol/L of random hexanucleotide primer, 1 mmol/L dNTP, 8 mmol/L $MgCl_2$, 30 mmol/L KCl, 50 mmol/L Tris-HCl, pH 8.5, 0.2 mmol/L ditiothreitol, 25 U RNAse inhibitor, and 40 U AMV reverse transcriptase. The mixture was incubated at 30℃ for 10 minutes and 42℃ for one hour, followed by inactivation of the enzyme at 99℃ for five minutes.

PCR amplification was performed at a final concentration of 1 × PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$), 20 μmol/L dNTP, sense and antisense primers (20 pmol for GAPDH; 30 pmol for iNOS), Taq polymerase (1U for GAPDH) or Taq-Gold polymerase (1.5 U for iNOS) in a total volume of 50 μL. Sense primer for iNOS was 5′-GGTGAAAGCGGTGT TCTTTGC-3′, and antisense primer was 5′-GGTG AGACAGTTTCTGGTCG-3′, which yielded a 389 bp PCR product. Sense primer for GAPDH was 5′-GACAAGATGGTGAAGGTCGG-3′, and antisense primer was 5′-CATGGACTGTGGTCATGA AGC-3′, which yielded a 538 bp PCR product. The PCR conditions were as follows: (1) GAPDH, 38 cycles, denaturating at 94℃ for 30 seconds, annealing at 60℃ for 30 seconds, and extension at 72℃ for 1 minute; (2) iNOS, 43 cycles, denaturating at 94.5℃ for 1 minute, annealing at 60℃ for 1 minute, and extension at 72℃ for 2 minutes. Initial heating at 95℃ for 9 minutes, and final extension at 72℃ for 7 minutes, were performed for all PCRs. Each cDNA was amplified with 30 to 50 cycles using specific primers, and the amplification cycles were selected during the exponential phase. The RT-PCR products were separated by electrophoresis, and the band densities analyzed by laser densitometry (Helena Laboratories, Beaumont, TX, USA).

Western blot analysis

Isolated rat glomeruli were lyzed in a sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mmol/L Tris-HCl, pH 6.8, 10% (vol/vol) glycerol]. The lysate was centrifuged at 12,000 × g for 15 minutes at 4℃, and the supernatant stored at -70℃ until all the rats were sacrificed. Protein concentrations were determined with a Bio-Rad kit. Aliquots, 30 μg of protein, were mixed with sample buffer, containing SDS and β-mercaptoethanol, heated at 100℃ for five minutes, and underwent electrophoresis in a 7.5% acrylamide denaturing SDS-polyacrylamide gel. Proteins were transferred to a Hybond-ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA) using a Hoeffer semidry blotting apparatus (Hoeffer instruments, San Francisco, CA, USA). The blots were then blocked, using 5% non-fat milk, and probed with iNOS-specific (mouse monoclonal anti-rat antibody; Transduction Laboratories, Lexington, KY) antibody for 1 hour at room temperature. The membrane was washed with PBS-Tween-20 for 1 hour, then probed with peroxidase-conjugated secondary antibody (horseradish peroxidase-linked anti-mouse IgG; Santa Cruz, CA, USA) for 1 hour at room temperature. After washing, immunoblots of the iNOS protein were visualized by enhanced chemiluminescence (Amersham Life Science, Little Chalfont, England, UK). Positive immunoreactive bands were quantified densitometrically.

Immunohistochemistry

Deparaffinized and rehydrated 4 μm thick sections were treated with 3% H_2O_2, to block endogenous peroxidase, and incubated with normal blocking serum, for 30 minutes, to block nonspecific staining. Sections were incubated for 1 hour at room temperature, and then overnight at 4℃ in a humidity chamber with polyclonal rabbit anti-mouse iNOS antibody (1:500 dilution, Affinity Bioreagents, Golden, CO, USA). After washing in PBS (pH=7.5), the sections were sequentially incubated with biotinylated link antibody and

peroxidase-labeled streptavidine (Dako, Santa Barbara, CA, USA). The substrate reaction was carried out using 3-amino-9-ethylcarbazole. The sections were counterstained with hematoxylin and mounted in an aqueous mounting media. As a positive control, sections with pyelonephritis were used. For a negative control, PBS was replaced with primary antibody.

Statistical analysis

Results are presented as the mean ± standard error (SE). The means between the two groups were compared using the Mann-Whitney U-test. Analysis of variance was used to assess the differences between multiple groups. A p-value < 0.05 was used as the criterion for a statistically significant difference.

RESULTS

Clinical parameters

Tables 1 and 2 show the time course data of clinical parameters. Compared with the C group, the mean blood glucose levels of the D rats were significantly higher throughout the 12-week period. The C rats gained weight over the 12-week experiment, however, the D rats failed to gain weight. The ratio of kidney to body weight was significantly higher in the D rats. The treatment with I or L did not affect these parameters. SBP in the D rats rose significantly at week 4 compared with the C rats, and kept significantly higher levels throughout the observation period of 12 weeks. The treatment with I or L, at the dose administered in this study, resulted in no significant reduction in SBP compared with that in the untreated D rats.

Twenty four-hour UPE was significantly greater in the D rats over the 12-week experiment. The treatment with I or L partly reversed this enhancement of UPE in the D rats.

Expression of iNOS mRNA and protein

Without LPS stimulation, the iNOS mRNA and protein expression were not apparent at week 4 and 8, but were found at week 12. At 12 weeks, the basal expression of glomerular iNOS mRNA was increased in the D rats compared with that of the C rats by RT-PCR (Fig. 1), whereas the Western blot analyses showed no significant difference in the level of protein between the two groups (Fig. 2).

Upon LPS stimulation, significantly higher level of iNOS mRNA expression in the D group was noted from week 4 (1.29 ×), and was maintained throughout the observation period (2.70 × at

Table 1. Blood Glucose, Body Weight, and Kidney Weight/Body Weight in 4 Groups

Parameter	Control	DM	DM+I	DM+L
Blood glucose (mg/dl)				
Week 0	93.4 ± 1.3	480.3 ± 6.1[a]	487.3 ± 7.8[a]	477.0 ± 6.0[a]
Week 4	117.3 ± 5.1	468.7 ± 9.7[a]	462.0 ± 13.1[a]	473.5 ± 25.5[a]
Week 8	138.7 ± 6.5	465.0 ± 11.2[a]	463.5 ± 18.3[a]	458.0 ± 16.0[a]
Week 12	121.1 ± 8.1	495.5 ± 1.2[a]	498.0 ± 2.9[a]	486.0 ± 12.0[a]
Body weight (g)				
Week 0	252.9 ± 4.7	261.1 ± 4.1	269.8 ± 4.7	277.0 ± 3.0
Week 4	397.7 ± 5.5	250.8 ± 8.0[a]	285.8 ± 17.1[a]	297.03 ± 3.0[a]
Week 8	471.2 ± 11.4	258.1 ± 11.8[a]	264.6 ± 26.8[a]	296.5 ± 55.5[a]
Week 12	510.3 ± 24.2	266.3 ± 18.5[a]	258.8 ± 34.0[a]	284.5 ± 91.5[a]
Kidney weight / Body weight (× 1000)				
Week 4	7.1 ± 0.3	15.3 ± 0.4[a]	–	–
Week 8	6.2 ± 0.2	15.2 ± 0.8[a]	–	–
Week 12	6.5 ± 0.2	15.1 ± 0.3[a]	16.8 ± 2.0[a]	14.3 ± 2.3[a]

Data are expressed as mean±SEM, I: imidapril, L: L-158,809, [a]p <0.01 vs. control.

Table 2. Systolic Blood Pressure and 24-Hour Urinary Protein Excretion in 4 Groups

Parameter	Control	DM	DM+I	DM+L
SBP (mmHg)				
Week 0	113.5 ± 3.0	115.5 ± 5.6	113.5 ± 5.7	108.6 ± 11.1
Week 4	122.7 ± 2.9	132.4 ± 3.1a	130.1 ± 11.1	132.5 ± 23.8
Week 8	122.2 ± 1.7	136.9 ± 4.0[a]	136.6 ± 3.4[a]	137.2 ± 2.9
Week 12	131.5 ± 4.9	148.1 ± 5.4[a]	137.3 ± 6.8	142.0 ± 1.7
Urinary protein excretion (mg/d)				
Week 4	23.0 ± 1.5	78.8 ± 7.2[a]	45.4 ± 1.9[a,b]	66.3 ± 6.0[a]
Week 8	23.9 ± 1.2	73.8 ± 7.2[a]	50.2 ± 3.7[a,b]	62.5 ± 3.6[a]
Week 12	19.8 ± 3.2	90.6 ± 11.3[a]	59.0 ± 2.8[a,b]	61.6 ± 6.6[a,b]

Data are expressed as mean±SEM.
I: imidapril, L: L-158,809, [a]$p<0.05$ vs. control, [b]$p<0.05$ vs. DM.

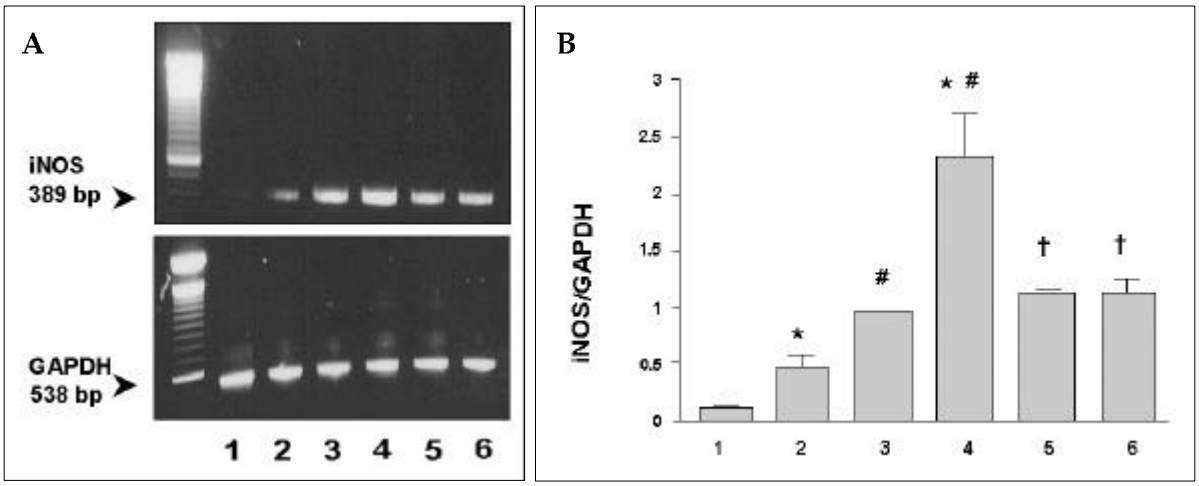

Fig. 1. (A) A representative RT-PCR at 12 weeks. (B) Values are the intensity of densitometric readings of iNOS mRNA corrected for GAPDH mRNA. Lane 1, control (C); lane 2, DM; lane 3, C + LPS; lane 4, DM + LPS; lane 5, DM + LPS + I; lane 6, DM + LPS + L. Values are expressed as a relative to that of C + LPS from four experiments. I: imidapril, L: L-158,809. *$p<0.05$ vs. paired C; #$p<0.05$ vs. corresponding values without LPS; †$p<0.05$ vs. DM+LPS.

week 8, 2.42 × at week 12) compared with the corresponding C group (Fig. 3). Administration of I or L in the D rats attenuated iNOS mRNA expression (1.12 ×, 1.12 × of the C group) at week 12 (Fig. 1). At the level of protein, D glomeruli expressed significantly higher levels of iNOS at week 8 (6.16 ×) and 12 (1.86 ×) compared with the corresponding C group (Fig. 3). Administration of I (0.77 × of the C group) or L (0.94 × of the C group) for 12 weeks, restored the enhanced expression of the iNOS protein observed in the D glomeruli compared to that seen in the glomeruli from the C rats (Fig. 2).

Histology and IHC staining for iNOS protein

Cytoplasmic clear cell change of the distal tubular epithelium was present in the D rats regardless of LPS treatment from week 4 onwards. The glomeruli of the D rats were hypertrophied compared with the C rats, but were normocellular. Mesangiolysis was present in five of the twenty LPS-treated D rats.

There was negative staining for iNOS within the glomeruli of five C rats without LPS treatment, but one of four rats with LPS treatment showed minimal iNOS staining in the glomeruli. In the D rats, the glomerular mesangium and

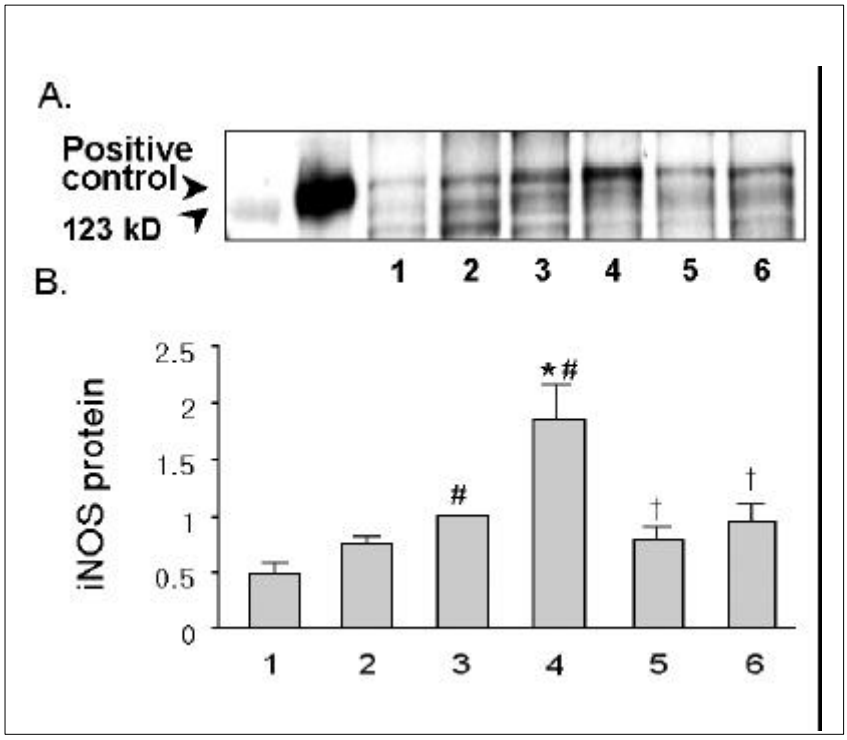

Fig. 2. (A) A representative Western blot at 12 weeks. (B) Values are the intensity of densitometric readings of iNOS protein. Lane 1, control(C); lane 2, DM; lane 3, C + LPS; lane 4, DM + LPS; lane 5, DM + LPS + I; lane 6, DM + LPS + L. Values are expressed as a relative to that of C+LPS from four experiments. I: imidapril, L: L-158,809. $^{*}p<0.05$ vs. C + LPS; $\#p<0.05$ vs. corresponding values without LPS; $^{\dagger}p<0.05$ vs. DM + LPS.

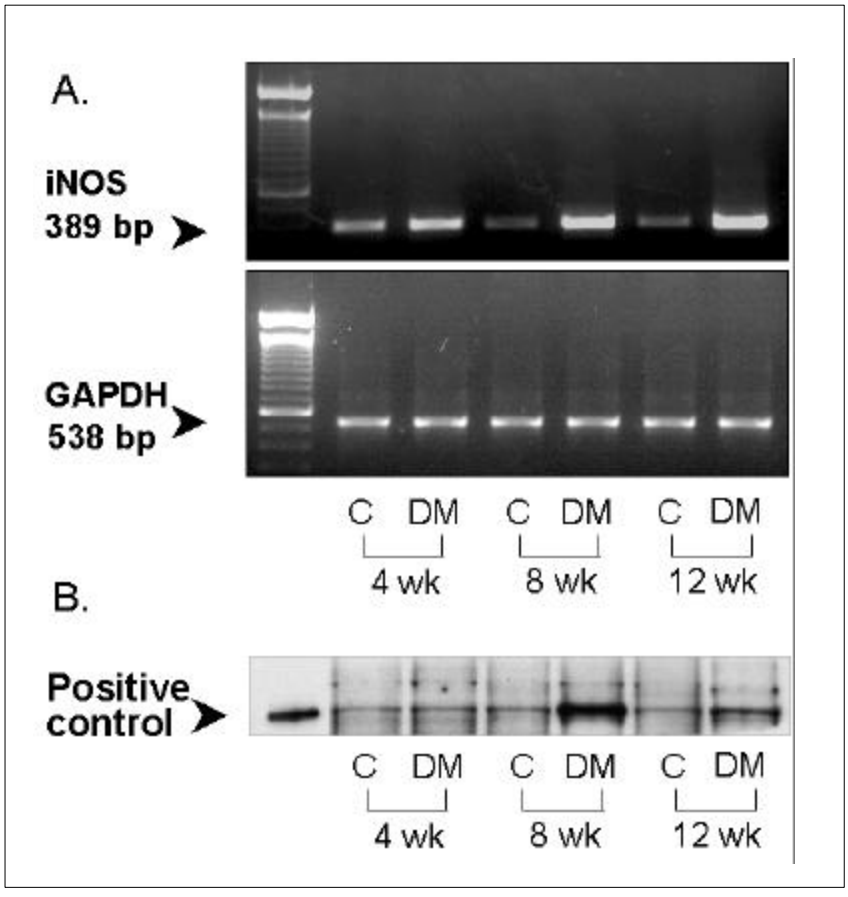

Fig. 3. (A) A representative RT-PCR of four separate experiments at indicated times. There was 1.29-fold, 2.70-fold and 2.42-fold increases in glomerular iNOS mRNA expression in DM rats compared with corresponding control rats at 4, 8 and 12 weeks. (B) A representative iNOS Western blot of four separate experiments at indicated times. Densitometric quantitation revealed that there was 6.16-fold and 1.86-fold increases in DM rats compared with corresponding control rats at 8 and 12 weeks. All the rats of these experiment were treated with LPS.

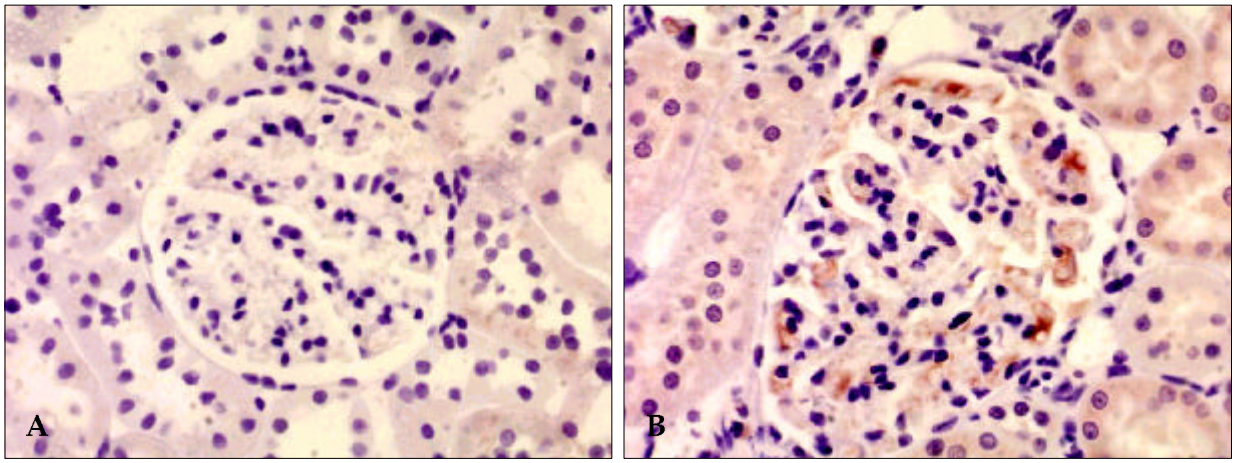

Fig. 4. Immunohistochemical staining for iNOS is negative in a glomerulus of control rats (A), but positive in the mesangium and capillary loop of diabetic rats (B).

podocytes were positive for iNOS in three of the five rats with, and without, LPS treatment (Fig. 4). No rats showed more than 10% positivity of the glomeruli.

DISCUSSION

Although several studies have suggested the alteration of the NO system could contribute to the pathogenesis of DM nephropathy,[9-13] they primarily focused on the constitutive NOS (cNOS) pathway, with the role of the iNOS pathway remaining unclear. It was logical to consider the possibility that iNOS may be involved in the pathogenesis of DM nephropathy because iNOS is increased in the beta cells of the pancreas after streptozotocin treatment,[14] and high glucose concentrations have been demonstrated to increase iNOS mRNA expression in macrophages and glomerular MCs.[6-7]

Our study shows that LPS-stimulated iNOS expression is increased in diabetic glomeruli at the levels of mRNA and protein. Since previous studies described the expression of iNOS was not detected in the basal condition,[15] and also reported enhanced production of a variety of cytokines including TNF-α and IFN-γ in diabetes,[3] the present study examined iNOS expression with, and without, LPS stimulation. The basal expression of iNOS mRNA and protein was not apparent at 4 and 8 weeks but was found at week 12. Although the Western blot analyses have not confirmed the difference in basal expression of iNOS protein between C and D glomeruli, the results of the RT-PCR and IHC suggests a greater expression of iNOS in D glomeruli compared with that of the C. With LPS stimulation, the D rats had more iNOS mRNA and protein expression from 4 weeks onwards. Recently, Sugimoto et al. reported that the expression of iNOS significantly increased in the D rat glomeruli at 52 weeks after the induction of diabetes.[16] They suggested the sequential pathway of advanced glycation end- products (AGE)-cytokine-iNOS in the development of diabetic nephropathy. Because we stimulated the rats with LPS in the early stages of diabetes, the role of AGE in the induction of iNOS was not evaluated in this study. The results of this study have confirmed our earlier *in vitro* study, in which exposure of rat MCs to high glucose enhanced cytokine-induced iNOS mRNA and protein with increased NO production.[7]

Angiotensin II leads to renal vasoconstriction, regulating glomerular perfusion, as well as filtration, and directly influences the mesangial constrictive response.[17] Angiotensin II is a potent regulator of MC phenotypical changes, such as migration,[18] proliferation,[19] or protein kinase C expression.[20] This supports its role as a growth factor, profibrogenic and proinflammatory media-

tor,[21] and regulator of additional proinflammatory mediators, such as: transforming growth factor-β (TGF-β)[22] or monocyte-chemoattractant protein-1 (MCP-1).[23]

Angiotensin II and NO have been postulated to interact closely. Conflicting data has been published regarding the effect of anigotensin II on iNOS expression in various experimental models. Some authors report a down-regulation, or inhibition, of the induced NO production in cultured rat astroglial cells by certain proinflammatory stimuli,[24] and similarly, in renal cells such; as mouse proximal tubular cells[25] and rat MCs.[26] Others were able to demonstrate an augmented LPS- or cytokine-induced NO production in rat cardiac myocytes.[27-28] Mehta et al. reported that the manipulation of the renin-angiotensin system, with antisense-oligodeoxynucleotides directed at ACE mRNA, inhibits the expression of the iNOS in the myocardial ischemia model.[29]

Recently, Schwobel et al. reported that angiotensin II receptor subtypes determine the influence of angiotensin II on the production of inducible NO in rat MCs.[30] They demonstrated that induced NO production is negatively controlled by the angiotensin II type 2 receptor (AT2), whereas AT1 stimulation enhanced NO synthesis in MCs. Our data shows that the up-regulation of LPS-stimulated iNOS expression was ameliorated by ACE inhibitor or AT1 blocker, at the levels of mRNA and protein, suggesting angiotensin II mediates the enhancement of iNOS expression via AT1 in diabetic glomerular injury, and are in agreement with the work of Schwobel.[30] To our knowledge, this is the first study providing evidence of the linkage between angiotensin II and glomerular iNOS pathway in DM nephropathy.

It is possible the balance of cNOS/iNOS is more important than the levels of cNOS, or iNOS, themselves, in the pathophysiological conditions. Recently, Kobayashi et al.[31] and Bachetti et al.[32] reported, by maintaining a correct ratio between eNOS and iNOS, ACE inhibitors are likely to protect the endothelium, and reduce the progression of atherosclerosis. Also, NO has been demonstrated to exert the capacity to autoinhibit NOS via specific binding sites on the enzyme.[33] Schwarts et al. have shown that local overproduction of NO, by iNOS, may down-regulate cNOS by autoinhibition.[34] From the results of our studies, taken together with previously published data showing that constitutive NO production is reduced in DM nephropathy,[12-13] further studies are necessary to investigate the role of the cNOS/iNOS balance in the development of diabetic glomerular injury.

In summary, this study is the first report on the alteration of the iNOS pathway in diabetic glomerulopathy and the contribution of angiotensin II in the changes of the iNOS system. Based on the previous studies, showing the enhancement of the iNOS pathway may promote the accumulation of extracellular matrix in various experimental models,[7,35,36] these results may implicate the iNOS pathway as a potential mediator of DM nephropathy.

ACKNOWLEDGMENTS

The authors are thankful to Donga Pharmaceutical, Korea and MSD, Korea, for their generous supplies of imidapril and L-158,809.

REFERENCES

1. Craven PA, DeRubertis FR, Melhem M. Nitric oxide in diabetic nephropathy. Kidney Int 1997;52Suppl60:S46-53.
2. Star RA. Intrarenal localization of nitric oxide synthase isoforms and soluble guanylyl cyclase. Clin Exp Pharmacol Physiol 1997;24:607-10.
3. Nakamura T, Fukui M, Ebihara I, Osada S, Nagaoka I, Tomino Y, et al. mRNA expression of growth factors in glomeruli from diabetic rats. Diabetes 1993;42:450-6.
4. Young B, Johnson R, Alpers C, Eng E, Floege J, Couser W. Mesangial cell proliferation precedes development of glomerulosclerosis in experimental diabetic nephropathy[Abstract]. J Am Soc Nephrol 1992;3:770.
5. Trachtman H, Futterweit S, Crimmins DL. High glucose inhibits nitric oxide production in cultured rat mesangial cells. J Am Soc Nephrol 1997;8:1276-82.
6. Sharma K, Danoff TM, DePiero A, Ziyadeh FN. Enhanced expression of inducible nitric oxide synthase in murine macrophages and glomerular mesangial cells by elevated glucose levels: Possible mediation via protein kinase C. Biochem Biophys Res Commun 1995; 207:80-8.
7. Noh H, Ha H, Yu MR, Kang SW, Choi KH, Han DS, et al. High glucose increases inducible NO production

in cultured rat mesnagial cells: Possible role in fibronectin production. Nephron 2002;90:78-85.
8. Kreisberg JI, Hoover RL, Karnovsky MJ. Isolation and characterization of rat glomerular epithelial cells *in vitro*. Kidney Int 1978;14:21-30.
9. Bank N, Aynedjian HS. Role of EDRF (nitric oxide) in diabetic renal hyperfiltration. Kidney Int 1993;43:1306-12.
10. Komers R, Allen TJ, Cooper ME. Role of endothelium-derived nitric oxide in the pathogenesis of the renal hemodynamic changes of experimental diabetes. Diabetes 1994;43:1190-7.
11. Tolins JP, Shultz PJ, Raij L, Brown DM, Mauer SM. Abnormal renal hemodynamic response to reduced renal perfusion pressure in diabetic rats: role of NO. Am J Physiol 1993;265:F886-95.
12. Wang YX, Brooks DP, Edwards RM. Attenuated glomerular cGMP production and renal vasodilation in streptozotocin-induced diabetic rats. Am J Physiol 1993;264:R952-6.
13. Craven PA, Studer RK, DeRubertis FR. Impaired nitric oxide-dependent cyclic guanosine monophosphate generation in glomeruli from diabetic rats: Evidence for protein kinase C-mediated suppression of the cholinergic response. J Clin Invest 1994;93:311-20.
14. Rabinovitch A, Suarez-Pinzon WL, Sorensen O, Bleackley RC. Inducible nitric oxide synthase (iNOS) in pancreatic islets of nonobese diabetic mice: identification of iNOS-expressing cells and relationships to cytokines expressed in the islets. Endocrinology 1996;137: 2093-9.
15. Veelken R, Hilgers KF, Hartner A, Haas A, Bohmer KP, Sterzel RB. Nitric oxide synthase isoforms and glomerular hyperfiltration in early diabetic nephropathy. J Am Soc Nephrol 2000;11:71-9.
16. Sugimoto H, Shikata K, Wada J, Horiuchi S, Makino H. Advanced glycation end products-cytokine-nitric oxide sequence pathway in the development of diabetic nephropathy: aminoguanidine ameliorates the overexpression of tumor necrosis factor-α and inducible nitric oxide synthase in diabetic rat glomeruli. Diabetologia 1999;42:878-86.
17. Schlatter E, Ankorina I, Haxelmans S, Kleta R. Effects of diadenosine polyphosphates, ATP and angiotensin II on cytosolic Ca^{2+} activity and contraction of rat mesangial cells. Pflugers Arch 1995;430:721-8.
18. Kohno M, Yasunari K, Minami M, Kano H, Maeda K, Mandal AK, et al. Regulation of rat mesangial cell migration by platelet-derived growth factor, angiotensin II, and adrenomedullin. J Am Soc Nephrol 1999;10: 2495-502.
19. Huwiler A, Stabel S, Fabbro D, Pfeilschifter J. Platelet-derived growth factor and angiotensin II stimulate the mitogen-activated protein kinase cascade in renal mesangial cells: comparison of hypertrophic and hyperplastic agonists. Biochem J 1995;305:777-84.
20. Amiri F, Garcia R. Regulation of angiotensin II receptors and PKC isoforms by glucose in rat mesangial cells. Am J Phyiol Renal Physiol 1999;276:F691-9.
21. Wolf G. Angiotensin II is involved in the progression of renal disease: importance of non-hemodynamic mechanisms. Nephrologie 1998;19:451-6.
22. Zoja C, Abbate M, Corna D, Capitanio M, Donadelli R, Bruzzi I, et al. Pharmacologic control of angiotensin II ameliorates renal disease while reducing renal TGF-beta in experimental mesangioproliferative glomerulonephritis. Am J Kidney Dis 1998;31:453-63.
23. Wolf G, Schneider A, Helmchen U, Stahl RA. AT1-receptor antagonists abolish glomerular MCP-1 expression in a model of mesangial proliferative glomerulonephritis. Exp Nephrol 1998;6:112-20.
24. Kopnisky KL, Sumners C. Angiotensin II-induced decrease in expression of inducible nitric oxide synthase in rat astroglial cultures: role of protein kinase C. J Neurochem 2000;74:613-20.
25. Wolf G, Ziyadeh FN, Schroeder R, Stahl RA. Angiotensin II inhibits inducible nitric oxide synthase in tubular MCT cells by a posttranscriptional mechanism. J Am Soc Nephrol 1997;8:551-7.
26. Kihara M, Yabana M, Toya Y, Kobayashi S, Fujita T, Iwamoto T, et al. Angiotensin II inhibits interleukin-1 beta-induced nitric oxide production in cultured rat mesnagial cells. Kidney Int 1999;55:1277-83.
27. Ikeda U, Maeda Y, Kawahara Y, Yokoyama M, Shimada K. Angiotensin II augments cytokine-stimulated nitric oxide synthesis in rat myocytes. Circulation 1995;92:2683-9.
28. Yasuda S, Lew WY. Angiotensin II exacerbates lipopolysaccharide-induced contractile depression in rabbit cardiac myocytes. Am J Physiol Heart Circ Physiol 1999;276:H1442-9.
29. Mehta JL, Chen H, Li D, Phillpis IM. Modulation of myocardial SOD and iNOS during ischemia-reperfusion by antisense directed at ACE mRNA. J Mol Cell Cardiol 2000;32:2259-68.
30. Schwobel J, Fischer T, Lanz B, Mohaupt M. Angiotensin II receptor subtypes determine induced NO production in rat glomerular mesangial cells. Am J Physiol Renal Physiol 2000;279:F1092-100.
31. Kobayashi N, Higashi T, Hara K, Shirataki H, Matsuoka H. Effects of imidapril on NOS expression and myocardial remodeling in failing heart of Dahl salt-sensitive hypertensive rats. Cardiovasc Res 1999; 44:518-26.
32. Bachetti T, Comini L, Pasini E, Cargnoni A, Curello S, Ferrari R. Ace-inhibition with quinapril modulates the nitric oxide pathway in normotensive rats. J Mol Cell Cardiol 2001;33:395-403.
33. Rengasamy A, Johns RA. Regulation of nitric oxide synthase by nitric oxide. Mol Pharmacol 1993;44:124-8.
34. Schwartz D, Mendonca M, Schwartz I, Xia Y, Satriano J, Wilson CB, et al. Inhibition of constitutive nitric oxide synthase (NOS) by nitric oxide generated by inducible NOS after lipopolysaccharide administration provokes renal dysfunction in rats. J Clin Invest 1997; 100:439-48.

35. Narita I, Border WA, Ketteler M, Noble NA. Nitric oxide mediates immunologic injury to kidney mesangium in experimental glomerulonephritis. Lab Invest 1995;72:17-24.
36. Yang CW, Yu CC, Ko YC, Huang CC. Aminoguanidine reduces glomerular inducible nitric oxide synthase (iNOS) and transforming growth factor-beta1 (TGF-β1) mRNA expression and diminishes glomerulosclerosis in NZB/W F1 mice. Clin Exp Immunol 1998;113:258-64.