Gene Expression of Intrarenal Renin-angiotensin System in Streptozotocin-induced Diabetic Rats

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In humans and many animal models with chronic progressive renal diseases, angiotensin-converting enzyme (ACE) inhibitor markedly attenuates the progression of nephropathy. Several studies have reported augmented gene expression and redistribution of renal renin in partial nephrectomized rats. Although precise mechanism(s) is not known, the renin-angiotensin system (RAS) may play an important role in the progression of renal diseases.

Thus, this study was undertaken to examine the gene expression of renal renin, angiotensinogen, and AT_1 subtypes (AT_{1A} and AT_{1B}) in rats with diabetic nephropathy, and the influences of lipopolysaccharide (LPS)-induced septicemia on the gene expression. Four weeks after streptozotocin (STZ) treatment (55 mg/kg, i.p.), rats were randomly divided into LPS-treated (1.6 mg/kg, i.p.) and control rats. At 6 hours after LPS treatment, the rats were killed and the kidney was removed from each rat. Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) techniques were used to detect mRNA expression. STZ treatment markedly attenuated body weight gain and significantly increased blood glucose level. Renal renin content (RRC) was significantly decreased in the STZ-treated rats compared to that in control rats. The renal ACE activity between STZ-treated and control rats was not significantly different. Renal renin mRNA level was prominently increased, while angiotensinogen and AT_{1A} mRNA levels were slightly decreased in STZ-treated rats compared to those in controls. AT_{1B} mRNA level did not differ in both groups. Acute LPS treatment did not show any significant changes of mRNA levels of intrarenal RAS components in both groups.

These results suggest that intrarenal RAS components were differentially regulated in STZ-treated diabetic rats. Further studies are required to evaluate the relationship between intrarenal RAS and other vasomodulatory systems.

Key Words: Renin-angiotensin system, Diabetic nephropathy, Northern blot, RT-PCR

INTRODUCTION

Chronic diabetes mellitus is frequently complicated by hypertension and renal disease (Krolewski et al, 1985; Viberti, 1988). Diabetic nephropathy constitutes a major cause of morbidity and mortality among diabetic patients (Andersen et al, 1983; Krolewski et al, 1987). Renal hemodynamic alterations play an important pathogenetic role in the initiation and pro-

gression of diabetic nephropathy (Hostetter et al, 1982; Zatz et al, 1985; Anderson et al, 1989). Shortly after the onset of diabetes there is an elevation in glomerular filtration rate (GFR). GFR subsequently returns to normal or progressively declines to result in chronic renal failure (Hostetter et al, 1982; Parving et al, 1983).

The renin-angiotensin system (RAS) has an important role in the physiologic regulation of the renal microcirculation. Independent from its systemic counterpart, a locally expressed and regulated RAS in the kidney may be involved in the control of renal function (Dzau et al, 1988). Abnormalities in RAS

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have been described in diabetes mellitus that may be causally related to the pathogenesis of the glomerulonephropathy (Hostetter et al, 1982). A detailed analysis of the dynamics of glomerular ultrafiltration in diabetic rats has demonstrated that hyperfiltration results from reduced arteriolar resistance that leads to elevated glomerular capillary plasma flow and increased transmural hydrostatic pressure differences across the capillary (Hostetter et al, 1981). In conjunction with other vasoactive systems, the RAS may contribute to the imbalance of resistances present at the preglomerular and postglomerular sites which are responsible for glomerular capillary hypertension, a major injurious factor in the diabetic kidney (Zatz et al, 1985; Anderson et al, 1989). Besides, angiotensin converting enzyme (ACE) inhibitors appear to provide a protective effect against glomerular and microvascular complications of the diabetic state (Anderson et al, 1989).

Nevertheless, the relationship between experimental and clinical diabetes and the RAS is unclear and the available reports vary widely (Anderson et al, 1990; Sajid et al, 1990; Sechi et al, 1990; Jaffa et al, 1991; Everett et al, 1992). In general, plasma renin activity (PRA) has been reported to be low in diabetic nephropathy (Christlieb, 1974; Burden & Thurston, 1979), although some investigators have found elevations in PRA in diabetic patients with glomerular hyperfiltration (Wiseman et al, 1984; Solerte et al, 1987). In spite of substantial evidence that the circulating RAS is depressed, local RAS activity may increase in diabetes. Therefore, this study examined the intrarenal RAS components in streptozotocin (STZ)-induced experimental diabetes. We measured renal renin content (RRC), renal ACE activity as well as the levels of renin, angiotensinogen and AT_1 receptor subtype mRNAs in the diabetic rats. To minimize the interexperimental variations, Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) were done in the kidney from a single set of animals.

Shultz and Raij (1992) demonstrated that the serum and urinary excretion of NO_2/NO_3 and nitric oxide (NO) synthesis were increased in rats given lipopolysaccharide (LPS) of gram-negative bacteria in vivo. In addition, Raij et al (1996) suggested modulation of the L-arginine-NO pathway may be important for the prevention as well as the treatment of many forms of renal disease. Therefore, we examined the effect of LPS-induced septicemia on the regulation of intrarenal RAS during diabetic nephropathy.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250~300 g) were used in all studies. Rats were made diabetic by a single intraperitoneal injection of 65 mg/Kg STZ (Sigma, ST. Louis, MO) made up fresh in sodium citrate buffer (pH 4.0). Control rats received the buffer only. Rats were maintained on standard laboratory rat chow and tap water ad libitum. Four weeks after the administration of STZ or vehicle, rats were randomly divided in LPS-treated (1.6 mg/kg, i. p.) and control rats. Then, rats were killed at six hours after LPS treatment. The kidney was removed from each rat and frozen in liquid nitrogen, and then kept in deep freezer ($-70°C$) until total RNA isolation.

Renal renin content (RRC) and angiotensin converting enzyme (ACE) activity

For the measurement of RRC, cortical slices (0.4 mm thick) were made in parallel to the renal surface, and the initial slice was discarded. Two slices, one from each renal hemisphere, were weighed and homogenized in 100 ml volume of cold 0.1 M Tris buffer, pH 7.4. The homogenate was centrifuged at 3,000 rpm for 30 min at 4°C and the supernatant was stored at $-20°C$ until assay. The supernatant, 10 μl, was incubated with excess angiotensinogen in the presence of protease inhibitors for 10 min at 37°C. Then a 20 μl aliquot was taken for radioimmunoassay (Lee et al, 1995) of generated angiotensin I (ANG I). Tissue renin content was expressed as μg ANG I · g protein^{-1} · h^{-1}.

Renal ACE activity was measured by colorimetric assay previously described by Neels et al. (1983). Briefly, the kidney was homogenized in 0.5 ml normal saline. Thereafter, 50 μl of homogenized sample was mixed with 100 μl of substrate solution (17 mM HGG, 30 mM HEPEG, 168 mM sodium chloride, 223 mM sodium sulfate, pH 8.15, 25°C) and incubated for 30 min at 37°C. The reaction was stopped by adding consecutively 100 μl of sodium tungstate (100 g/L) and 100 μl of dilute (0.33 M/L) H_2SO_4. Then, the sample was mixed well for 10 sec and 1,000 μl of water was added. Thereafter, it was centrifuged for 10 min at 2,000×g. 750 μl of the supernatant was moved to a new tube and 1,000 μl

of borate buffer (100 mM/L, pH 9.6) and 50 µl TNBS solution (60 mM/L) were added sequentially and mixed well. After incubation at 37°C for 15 min, the absorbance was read against a blank, prepared by adding the deproteinizing agents (sodium tungstate and H_2SO_4) to the substrate solution before the sample addition.

Total RNA isolation and Northern blot analysis

Total RNA was isolated according to the method described by Chomczynski et al (1987) with a slight modification. RNA pellet was redissolved in formamide and kept at $-20°C$ until needed. The concentration and purity of RNA was determined by measuring the absorbance at 260 nm and 280 nm.

Aliquots of total RNA (40 µg) were separated on a 1% agarose gel containing 0.66 M of formaldehyde in 1 X MOPS buffer (0.02 M MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, pH 8.0). RNA was transferred to a nitrocellulose membrane by capillary action in 20 X SSC (1 X SSC; 0.15 M sodium chloride, 0.015 M sodium citrate). The membrane was baked in a vacuum oven at 80°C for 2 h and prehybridized for 4 h at 42°C in a solution containing 50% formamide, 6 X SSC, 5 X Denhardt's solution (0.1% each of Ficoll 400, polyvinylpyrrolidine and nuclease-free bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS) and 0.1 mg/ml salmon sperm DNA. [α-^{32}P]dCTP-labeled angiotensinogen or renin

cDNA was added to the solution and the membrane was incubated for 18-20 h at 42°C. After hybridization, the membrane was sequentially washed in 2 X SSC, 0.2% SDS for 5 min at room temperature, for 30 min at 42°C. The membrane was washed thereafter in 0.1 X SSC, 0.1% SDS at 60°C until the signal and background were distinguishable. The resulting membrane was exposed to X-ray film for 1-4 days at $-70°C$.

Reverse transcription PCR (RT-PCR)

The nucleotide sequence of the primers and references are presented in Table 1. Twenty microgram of total RNA was primed with oligo(dT) primers, and first strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase in a 30 µl of reaction volume for 90 min at 37°C. The reaction mixture was heated for 5 min at 95°C to degrade template RNA and enzyme. PCR was carried out in a 25 µl reaction volume on DNA Thermal Cycler (Perkin-Elmer, Cetus). In brief, 1 or 2 µl of first strand cDNA reaction mixture was transferred to a thin-wall PCR tube and sterilized water was added to make the volume to 5 µl. To the reaction tube, 20 µl of PCR master mixture containing 10 X buffer (10 X polymerase standard buffer plus 5mM $MgCl_2$ and 0.1% formamide), 800 µM of dNTPs and 20 pmole of each primers was added to the tube. The reaction mixture in PCR tube was heated for 2 min at 95°C

Table 1. Primer sequences for AT_{1A}, AT_{1B}, renin and GAPDH used in this study

Gene	Primer sequence	Product size	Reference
AT_{1A}			
(S)	GCCTGCAAGTGAAGTGATTT	388 bp	Kitami et al [1992]
(AS)	TTTAACAGTGGCTTTGCTCC		
AT_{1B}			
(S)	GCACACTGGCAATGTAATGC	204 bp	Kitami et al [1992]
(AS)	GTTGAACAGAAGAAGTGACC		
renin			
(S)	AGGCAGTGACCCTCAACATTACCAG	362 bp	Burnham et al [1987]
(AS)	CCAGTATGCACAGGTCATCGTTCCT		
GAPDH			
(S)	ATCAAATGGGGTGATGCTGGTGCTG	504 bp	Tso et al [1985]
(AS)	CAGGTTTCTCCAGGCGGCATGTCAG		

Table 2. Weight gain, blood glucose concentration, renal renin content (RRC) and renal angiotensin converting enzyme (ACE) activity of rats 4 weeks after streptozotocin (STZ) treatment.

	Control	STZ
Weight gain (g)	108 ± 4.8	20 ± 8.2**
Blood glucose (mg/dl)	111 ± 9.8	485 ± 12.6**
RRC (μg ANGI · g protein^{-1} · h^{-1})	25.5 ± 3.4	16.6 ± 0.8**
ACE (U · mg protein^{-1} · L^{-1})	9.8 ± 1.2	11.0 ± 0.9

Values are mean ± SE of 5 rats.
** $p < 0.01$, vs. control groups.

then cyclic amplification profiles are as follows: 94°C for 45 sec, 54°C for 45 sec, 72°C for 1 min. PCR conditions are the same for all genes examined in this study except PCR cycles and the amount of template that were carefully selected according to the relative abundance of mRNAs in the kidney. After the end of PCR, one fifth of reaction mixture was separated on a 1.2% of agarose gel containing 0.5 μg/ml of ethidium bromide.

Densitometric analysis

The polaroid film was scanned using an Epson (ES-600) scanner with a resolution of 70 DPI (dot per inch) and 256 gray scale unit. In order to achieve a linear response curve, the brightness and the contrast was finely tuned to the point where the gray scale unit of the background were within 20-40 and that of the darkest band was between 200-240. The resulting image was analysed from NIH-Image analysis program (written by Wayne Rasband, NIH, Bethesda, MD). Using a selection box, the gray scale unit of various background areas were measured and subtracted from each band. Then, the mean density and total area of the band were measured. The scale of each band was expressed by multiplying the mean density and the total area of the band. The resulting scale was used to quantify each band.

Statistical analysis

All data are presented as mean ± SE. Unpaired *t*-test was used for the comparison of means of different groups. Differences were considered significant if $p < 0.05$.

RESULTS

The changes of body weight, blood glucose level, RRC and renal ACE activity are shown in Table 2. STZ treatment markedly attenuated body weight gain compared to that of control rats. However, we did not find out a weight loss. STZ treatment also induced a significant elevation of blood glucose level. RRC was significantly down-regulated in the STZ-treated rats compared to that in control rats. In contrast, renal ACE activity did not show any difference between STZ-treated and control rats. LPS treatment did not induce any acute changes in the above four parameters compared to that of LPS-untreated STZ rats (data not shown).

Renal renin and angiotensinogen mRNA levels were determined by Northern blot analysis. The size of renal renin and angiotensinogen mRNAs migrated on agarose gel electrophoresis were approximately 1.4 Kb and 1.6 Kb, respectively. Blots were then stripped and rehybridized to β actin probe. The level of β actin mRNA were unstable (data not shown) and we were not able to consistently measure the expression of β actin gene. Therefore, we analyzed the result of Northern blot of renin and angiotensinogen compared to 18 S and 28 S ribosomal RNA bands on ethidium bromide stained agarose gel. The level of renal renin mRNA was approximately 1.5- to 2.5-fold higher in the STZ-treated rats compared to that in control rats. We acquired the same result from the RT-PCR of renin (Fig. 3A and Fig. 4). The level of renal angiotensinogen mRNA was decreased to some extent in the STZ-treated rats than that in control rats (Fig. 2).
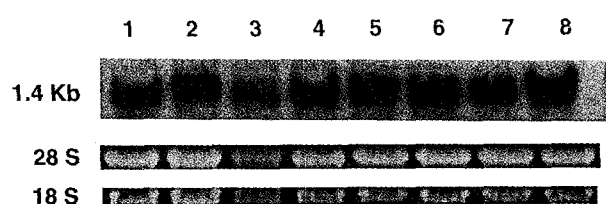
The results of RT-PCR for renin and AT$_1$ receptor

Fig. 1. Representative example of Northern blot analysis of renin mRNA. Total RNA (40 μg) was applied per lane as follows; control (lanes 1-2), control + LPS treatment (lanes 3-4), STZ treatment (lanes 5-6) and STZ + LPS treatments (lanes 7-8). Intact 28 S and 18 S ribosomal RNA bands are shown by ethidium bromide staining of agarose gel.
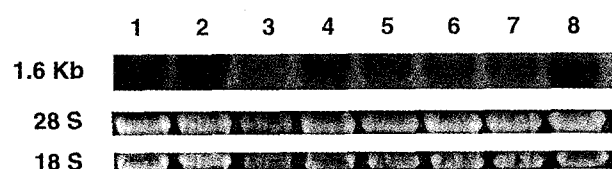

Fig. 2. Representative example of Northern blot analysis of angiotensinogen mRNA. Total RNA (40 μg) was applied per lane as follows; control (lanes 1-2), control + LPS treatment (lanes 3-4), STZ treatment (lanes 5-6) and STZ + LPS treatments (lanes 7-8) Intact 28 S and 18 S ribosomal RNA bands are shown by ethidium bromide staining of agarose gel.

Fig. 3. Ethidium bromide-stained gel of PCR products for renin (A), AT_{1A} (B) and AT_{1B} (C) mRNAs. PCR product (5 μl) was applied per lane as follows; control (lanes 1-2), control + LPS treatment (lanes 3-4), STZ treatment (lanes 5-6) and STZ + LPS treatments (lanes 7-8).

subtypes are shown in Fig. 3. In the preliminary experiments we determined parameters to obtain a correlation between serially diluted PCR products and their densitometric signals. AT_{1A} mRNA level (Fig. 3B and Fig. 4) tended to decrease in STZ-treated rats compared to that in controls, while AT_{1B} mRNA level (Fig. 3C and Fig. 4) did not differ in both groups. The expression pattern of mRNA for AT_{1A} corresponded to that of angiotensinogen. Unexpectedly, LPS treatment did not show any changes of gene expression of intrarenal RAS components in both control and STZ-treated rats (data not shown).

DISCUSSION

The present study demonstrated that the level of renin mRNA was increased while RRC was significantly decreased in the STZ treated rats than in control rats. The mechanisms responsible for changes in kidney renin gene and protein expression during
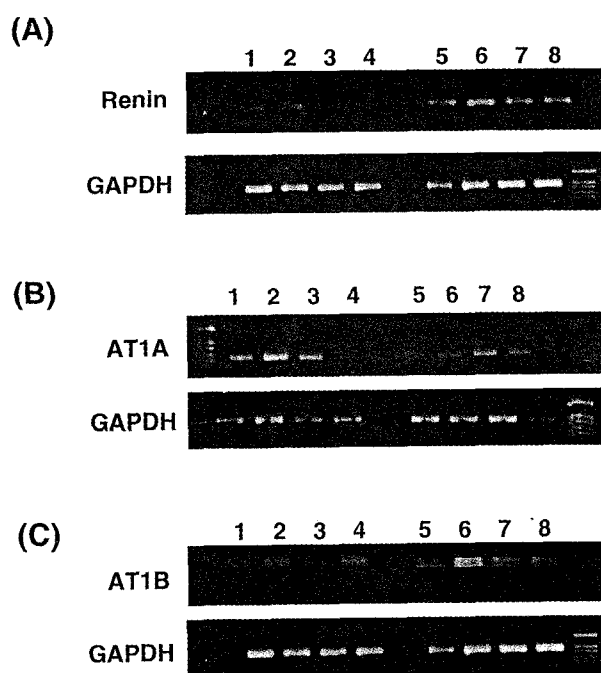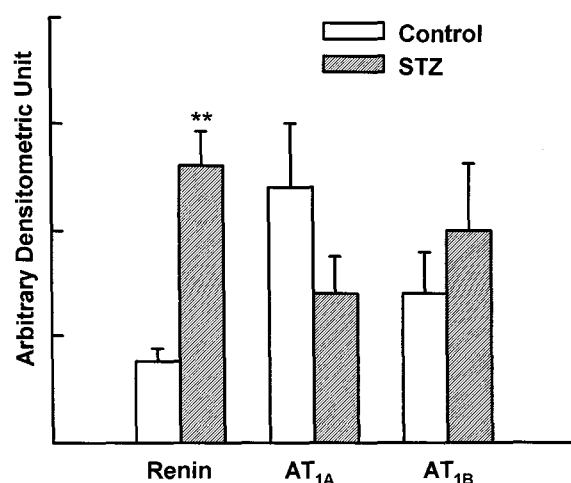

Fig. 4. Densitometric analysis of the PCR products for renin, AT_{1A} and AT_{1B} mRNAs corrected for GAPDH in each rat. The values were expressed in arbitrary densitometric unit. Control and STZ represent rats treated with vehicle and STZ, respectively. Values are mean ± SE of 4 rats. ** $p < 0.01$, vs. control groups.

diabetes are not well understood. Fluid and salt balance are known to affect the activity of the RAS (Davis & Freeman, 1976). In addition, low or high salt diets and excess volume expansion are known to alter plasma renin activity in the diabetic BB rat (Cohen et al, 1986; Katayama et al, 1990). The diabetic animals in this study were allowed free access to food and water. As expected, the diabetic rats had higher food intakes than the non-diabetic controls. Since both dietary sodium and protein modulate renin synthesis, the changes in renin may be influenced by these dietary components (Ingelfinger et al, 1986). The interaction between these dietary components and the diabetic state were not specifically dissected in this current study, but the pattern of results does not suggest that either increased protein intake or salt imbalance are solely responsible for the changes in the components of the RAS measured.

Multiple factors other than plasma volume (Ilstrup et al, 1981; Cohen et al, 1986) such as circulating atrial natriuretic peptide (Ortola et al, 1987) and prostaglandin synthesis (Craven et al, 1987) are altered during diabetes and are known to affect renin release and possibly synthesis in the nondiabetic animal. In addition, automatic regulation of renin release during diabetes may be impaired as reported in the BB rat (Cohen et al, 1986) and human (Fernandez-Cruz et al, 1981). Whether these physiological changes may in turn be responsible for alterations in renin gene transcription and/or mRNA stabilization during diabetes is not known and requires further study.

One possible explanation for the dissociation between the RRC and renin mRNA is the defect in conversion of prorenin to active renin in diabetes. Some reports suggested a decrease in prorenin conversion to active renin that could contribute to the increased plasma levels of prorenin demonstrated in diabetic rats and patients (Bryer-Ash et al, 1988;Sechi et al, 1990; Wilson & Luetscher, 1990) A prorenin to renin conversion defect could explain the lower active renin level in the face of normal to elevated levels of renal renin mRNA. A more distal change in renin secretion and/or increased peripheral renin clearance might also account for the lower renal renin content compared to mRNA levels. However, there is no clear reason to suspect the latter possibility (Kim et al, 1987). At present time, only limited data are available, and further studies are required to elucidate the mechanisms of dissociation between renin gene and protein

expression in the diabetic rats.

In the present study, we demonstrated the level of renin mRNA was significantly increased and, unlike renin, angiotensinogen gene expression appears to decrease in the STZ-treated rats. Preliminary studies examining the RAS in the diabetic kidney have yielded conflicting results. Anderson (1990) and coworkers reported increased renal renin content and angiotensinogen mRNA in insulin-treated diabetic rats six to eight weeks following STZ-induced diabetes. Sajid (1990) and collaborators examined renin mRNA was suppressed from one day to six weeks and then restored ro normal. Sechi (1990) and coworkers reported no change in angiotensinogen and renin mRNA in untreated diabetic rats compared to insulin-treated diabetic or control rats 12 days after STZ treatment. In spontaneously diabetic rats, Everett (1992) and coworkers demonstrated an initial increase in renal renin and angiotensinogen mRNA, and renin stained cells per juxtaglomerular apparatus. In addition, Jaffa (1991) and coworkers demonstrated that three weeks after the induction of diabetes with STZ, severely hyperglycemic rats had low renin mRNA while moderately hypergycemic rats had a renin mRNA level not different from control. All of these discrepancies are not readily explained given the preliminary nature of almost all of these reports. However, differences may be related to the varying time periods at which the rats were studied, the degree of glycemic control, dietary intake, and the diabetic model studied. Although the interpretation of these observations was not made due to limited data available, the results of this study suggest that the transcriptional and/or translational activity of renin and angiotensinogen may be regulated in an uncoordinated fashion and that different mechanisms may control the expression of the renal renin and angiotensinogen gene during diabetes.

Although quantitation of renin protein or mRNA does not provide definitive evidence for increased angiotensin II (ANG II) production, renin is the rate-limiting enzyme in the formation of ANG II. Furthermore, measurement of renal renin provides information about local renin synthesis in the kidney (Dzau et al, 1988). Definitive evidence for a pathogenetic role of the RAS rests on the results of studies examining specific blockade of this system. Studies of this nature suggest that the RAS is activated in diabetes (Anderson et al, 1989; Anderson et al, 1990). However, in the present study most components of

intrarenal RAS including renin content, angiotensinogen mRNA, AT_{1A} and AT_{1B} mRNAs are unchanged or significantly decreased in diabetic compared to non-diabetic animals. These results well corresponded to previous reports (Christlieb, 1974; Sajid et al, 1990; Sechi et al, 1990; Jaffa et al, 1991; Everett et al, 1992). This apparent discrepancy may be explained by post-receptor events. Alternatively, a number of other diverse vasoactive systems may be playing a role in the modulation of intrarenal hemodynamics in diabetes (Ortola et al, 1987; Craven et al, 1987). Therefore, even without remarkable elevation in most components of the intrarenal RAS did not occur, removing the effects of this system might have altered the critical balance between vasoconstrictor and vasodilator systems.

We demonstrated that AT_{1A} mRNA was slightly decreased while AT_{1B} mRNA was unchanged after STZ treatment. These results well corresponded to the ones observed previously. In the ventricle of spontaneously hypertensive rats, Iwai (1992) and coworkers showed the expression level of AT_{1B} mRNA was higher than that of age-matched Wistar-Kyoto rats, while the expression level of AT_{1A} mRNA was almost similar between the two strains at this age. They also have demonstrated the differential regulation of expression of these two subtypes in the adrenal gland and in the liver of the nephrectomized rats. Others (Kitami et al, 1992; Jo et al, 1996) also indicated similar differential regulation of expression of gene coding for both AT_1 subtypes.

Shultz and Raij (1992) demonstrated that the serum and urinary excretion of NO_2/NO_3 and nitric oxide (NO) synthesis were increased in rats given lipopolysaccharide (LPS) of gram-negative bacteria in vivo. In addition, they found out the glomeruli isolated from these rats continued to produce NO_2/NO_3 ex vivo tissue culture and L-N^G-nitroarginine methyl ester (L-NAME) inhibited in vivo and ex vivo synthesis of NO in response to LPS. In rats given LPS, inhibition of NO synthesis with L-NAME resulted in diffuse glomerular thrombosis. Several investigators (Moncada et al, 1991; Shultz & Raij, 1992) suggested endogenous synthesis of NO in sepsis and sepsis-related syndromes thus may play a role in maintaining renal vasodilation and inhibiting thrombus. Finally, Raij et al (1996) suggested modulation of the L-arginine-NO pathway may be important for the prevention as well as the treatment of many forms of renal disease. Therefore, we examined the effect of septicemia on the regulation of intrarenal RAS during diabetic nephropathy. However, we could not detect any remarkable change of renal RAS components after LPS treatment. Further studies are required to evaluate the relationship between intrarenal RAS and other local vasomodulatory systems.

In summary, these results suggest that intrarenal RAS components were differentially regulated in STZ-treated diabetic rats. Further studies are required to evaluate the relationship between intrarenal RAS and other vasomodulatory systems.

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