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Renoprotective effects of valsartan and enalapril in STZ-induced diabetes in rats

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Summary

Effects of the angiotensin II type 1 (AT_1) receptor antagonist valsartan and the angiotensin-converting enzyme (ACE) inhibitor enalapril were studied in streptozotocine (STZ)-induced diabetes in rats on the basis of microalbuminuria (Ma) and renal morphology. Five groups of Wistar rats were used, one group was the non-diabetic control, one group consisted of untreated STZ-diabetics and 3 groups of STZ-diabetics were treated with either enalapril and/or valsartan for 30 days. Blood glucose (BG) and Ma levels, body and kidney weight and glomerular size were measured. Immunohistochemical staining with an anti-transforming growth factor-β1 (TGF-β1) antibody was performed as well. In STZ-diabetics, BG and Ma levels were significantly increased when compared with the non-diabetic group. Although Ma levels in the valsartan-treated group was found to be higher than those in the non-diabetics group after 15 days of treatment, in all treated diabetic groups Ma levels were significantly decreased as compared with STZ-diabetics at the end of the experiment. Thickening of the glomerular and tubular basement membranes, increased mesangial matrix and glomerular size were found in the untreated diabetic group. All these changes were less in the treated groups. A significant increase in TGF-β1 immunoreactivity was found in glomeruli of untreated STZ-diabetics as compared with non-diabetics. Again, TGF-β1 expression was decreased in the treated groups as compared with untreated STZ-diabetics. We conclude that valsartan and enalapril have renoprotective effects in diabetic nephropathy**.** A combined therapy has an advantage because lower dosages of these drugs can be used. Their beneficial effects are related to a blockade of the renin-angiotensin system (RAS) and a decrease in TGF-β1 expression in glomeruli.

Key words: STZ-induced diabetes – enalapril – valsartan – TGF-β1 – rat

Introduction

Diabetic nephropathy is characterized by renal hypertrophy, thickening of glomerular and tubular basement membranes, increased amounts of extracellular matrix (ECM) in glomerular mesangium, tubular interstitium and increased glomerular permeability (Ziyadeh et al., 1991; Steffes et al., 1992). Microalbuminuria or evident proteinuria are the result of these changes in the kidney. Progress of the disease causes diffuse or nodular glomerulosclerosis, tubulointerstitial fibrosis, hyalinization in afferent and efferent arterioles, and loss of

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renal function resulting in end-stage renal insufficiency (Cooper and Gilbert, 2000).

TGF-β has been demonstrated to be a mediator of ECM deposition and hypertrophy in experimental diabetic and glomerular diseases (Gilbert et al., 1998; Nakamura et al., 1999; Hill et al., 2000). TGF-β is found in mammals in 3 isoforms: TGF-β1, 2, and 3. All these isoforms have similar effects. However, the TGF-β1 isoform is the most potent stimulator of fibrosis (Border and Noble, 1994). It has been shown in rats that expression of mRNA and protein of TGF-β1 and the synthesis of ECM proteins including collagen I and IV are both increased in STZ-induced diabetes (Nakamura et al., 1993; Sharma and Ziyadeh 1995; Gilbert et al., 1998; Hill et al., 2000). Moreover, Hill et al. (2001) have reported increased concentrations of TGF-β receptors in relation to increased levels of TGF-β isoforms in STZ-diabetic rats. Sharma et al. (1996) have shown that neutralization of TGF-β by an anti-TGF-β antibody attenuates the elevated ECM protein synthesis and renal hypertrophy in STZ-induced diabetes in mice.

Although compounds of the RAS are suppressed in plasma in diabetic nephropathy, their activation is continued locally in the kidneys. Hyperglycemia has a direct effect on the expression of RAS components in mesangial and proximal tubular cells (Zhang et al., 1999; Burns, 2000). The major active product of RAS is angiotensin II (AII). AII shows hemodynamic and trophic effects on the kidneys by activating the angiotensin II type 1 (AT_1) receptors (Mann, 1999; Burns, 2000). AII causes preferential vasoconstriction in efferent arterioles which results in intraglomerular hypertension and mesangial contraction. Riser et al. (1996) reported that TGF-β1 production and activation is increased in cultured mesangial cells exposed to cyclic stretching resulting in elevated amounts of ECM associated with intraglomerular hypertension. These phenomena may be explained by hemodynamic stimulation of TGF-β production and secretion. AII has been shown to stimulate production of TGF-β directly. Indeed, Kagami et al. (1994) have shown that TGF-β production and deposition of matrix proteins are increased when cultured rat mesangial cells are exposed to AII. This suggests that the effects of AII on matrix protein synthesis are dependent on autocrine effects of TGF-β released from mesangial cells and may not be dependent on hemodynamic effects of AII.

The aim of our study was to compare the effects of the AT_1 receptor antagonist, valsartan, and the ACE inhibitor, enalapril, in STZ-induced diabetes in rats by assessing microalbuminuria, renal morphology and renal TGF-β1 expression. We also determined whether combined use of valsartan and enalapril is superior over the use of these agents alone.

Material and methods

Animals and protocol

Female Wistar albino rats (n = 41) with initial body weight of 180–220 g were randomized over 5 groups. At the begining of the study, body weight and blood glucose levels were measured in all animals. The first group (n = 5) was the non-diabetic control group. In the other 4 groups (n = 9 in each group), one dose of 65 mg/kg STZ (Sigma, St. Louis MO, USA) in citrate buffer, pH 4.5, was given intraperitoneally. The second group was the untreated STZ-induced diabetic group. The third group was treated orally each day with 50 mg/kg valsartan (Novartis, Basel, Switzerland) dissolved in 1N KOH and 0.1N HCl, pH 7.2. The fourth group was treated orally with 10 mg/kg enalapril (Merck, Sharp and Dohm, İstanbul, Turkey) in 0.9% NaCl for 30 days. The fifth group was treated orally with a combination of enalapril (5 mg/kg), and valsartan (30 mg/kg) for 30 days. All groups were fed *ad libitum* with standard food pellet and tap water.

Blood glucose and microalbuminuria

The BG levels were measured weekly in blood taken from the tail vein of all rats by using glucostix (Bayer, İstanbul, Turkey) and a glucometer (Glucometer II Model 5550; Ames, Indianapolis IN, USA). The glucometer displays a “high” value when the blood glucose level is higher than 400 mg/dl. Animals were housed in metabolic cages and the Ma levels were measured at the first day and after every week during the study in samples of 24-h urine collection using a kit (Micral-Test II; Boehringer, İstanbul, Turkey).

Light microscopy

At the end of the study, rats were anesthetized with ether and renal tissue samples were obtained for morphological studies and fixed in Bouin’s fluid and then embedded in paraffin. Periodic acid Schiff (PAS) staining and immunohistochemical staining were used for histological examinations. For the calculation of glomerular size, 30 glomeruli from each PAS-stained section were selected randomly. Severely deformed glomeruli were excluded from calculations. Measurements were performed using a micrometric ocular system (Beck, Kassel, Germany).

Immunohistochemistry

Sections (5 µm thick) were placed onto slides coated with poly-L-lysine, 0.1% w/v in water, then deparaffinized and rehydrated. Immunoperoxidase staining was

performed using the ImmunoCruzTM Staining System kits including TGF-β1 antibody (Santa Cruz Biotechnology, Santa Cruz CA, USA). Immunostaining procedures were carried out following the guidelines of the manufacturer. Sections were incubated for 5 min with 1% hydrogen peroxide in PBS to block endogenous peroxidase activity. After washing the sections with PBS, incubation with normal blocking serum (goat serum) was performed. Sections were incubated overnight at 4 °C with prediluted anti-TGF-β1 rabbit polyclonal antibody, then washed with PBS and incubated with biotinylated secondary antibody (goat anti-rabbit-IgG), and finally washed with PBS. Then sections were incubated for 5–10 min with a substrate-chromogen solution (AEC; Zymed, San Francisco CA, USA) after incubation with horseradish peroxidase-streptavidin complex. Sections were counterstained with hematoxylin. To determine specificity of immunostaining, sections were incubated in the same way except for incubation with the primary antibody. Control serum (normal rabbit IgG) instead of the primary antibody was also used as control.

Semiquantitation of immunoperoxidase staining.

Immunoperoxidase staining of glomeruli was analysed and scored from 1+ to 4+ (1+, little immunopositivity to 4+, strong immunopositivity) using Hill's scoring system (Hill et al., 2000). This analysis was performed in a blind and randomized fashion of all TGF-β1-stained sections in at least 10 glomeruli per kidney section, in 2 sections from each animal and in 4 animals per group.

Statistics

Values were expressed as mean ± SD. Levels of BG and microalbuminuria, body weight, kidney weight of the 5 groups of rats were compared using Kruskal-Wallis one-way ANOVA and Friedman two-way ANOVA tests. The Dunn test was used for multiple comparisons. For evaluation of sizes of glomeruli, the one-way ANOVA and Tukey HSD test were used.

Results

Blood glucose levels, body and kidney weight

Rats became diabetic at 24 h following the STZ injection. Their BG levels were significantly higher than those of the non-diabetic control animals at this time point ($p < 0.01$). BG levels of enalapril-treated and enalapril and valsartan-treated STZ-diabetic groups were significantly lower than those of the untreated STZ-diabetic group at the end of the experiment ($p < 0.01$). BG levels in the valsartan-treated group were not different from those in the untreated STZ-diabetic group (Table 1). Body weight in the untreated STZ-diabetic group and all treated diabetic groups was decreased at the end of the experiment ($p < 0.01$; Table 2). However, a correlation between body weight loss and BG levels was not found. Kidney weight was not different in all groups (Table 3).

Microalbuminuria

After STZ-induced diabetes, Ma levels were significantly higher ($p < 0.01$) than in non-diabetic rats. At the end of the experiment, Ma levels were significantly lower in all treated diabetic rats than in non-treated diabetic rats ($p < 0.01$). Ma levels of enalapril-treated diabetic rats were similar to levels in the non-diabetic control group at 15 and 30 days after STZ injection. In valsartan-treated rats, Ma levels were higher than in the non-diabetic control group ($p < 0.001$) at day 15 but were similar to the levels in the non-diabetic control group at the end of the experiment. In animals treated with a combination of enalapril and valsartan, Ma levels were similar to the levels in control and enlapril-treated animals (Table 4).

Light microscopy

A significant enlargement of the glomeruli, thickening of glomerular and tubular basement membranes, increased amounts of mesangial matrix and tubular dilatation were observed in STZ-diabetic rats as compared with control animals (Figs. 1–3). Evidence for glomerulosclerosis and tubulointertitial fibrosis was never found. Renal tissue was less altered in all treated rats when compared with untreated STZ-diabetic rats, but focal dilated tubuli were still observed in enalapril-treated and valsartan-treated rats. In the treated STZ-diabetics, glomerular size was significantly reduced as compared with STZ-diabetics ($p < 0.05$; Table 3).

Immunohistochemistry

TGF-β1 immunostaining was intense in glomeruli of untreated STZ-diabetic rats as compared with non-diabetic controls. It was less intense in the treated diabetic rats (Figs. 2, 3; Table 5).

Discussion

Several studies have compared effects of ACE inhibitors and AT_1-receptor antagonists on renal hemodynamics, proteinuria and renal histology (Hutchison and Webster, 1992; Kohzuki et al., 1995a; Wu et al., 1997) but their mechanisms of action are still unclear.

Table 1. Blood glucose levels (mg/dl) in non-diabetic, untreated diabetic and diabetic rats treated with enelapril (E), valsartan (V) or a combination of E and V (E+V) before and after a single injection of STZ

Day	*Non-diabetic* (n = 5)	*STZ-diabetic* (n = 9)	*STZ-E* n = 9)	*STZ-V* (n = 9)	*STZ-E+V* (n = 9)
0	93.40 ± 8.41	88.22 ± 8.91	97.33 ± 11.33	100.55 ± 15.62	94.44 ± 12.11
1	97.60 ± 5.31	384.00 ± 34.30 [a]	390.44 ± 19.19 [a]	392.22 ± 18.95 [a]	378.77 ± 28.23 [a]
30	93.60 ± 14.70	400.00 ± 0.00 [b]	298.33 ± 71.20 [c]	357.00 ± 74.70 [b]	288.33 ± 93.13 [c]

Values are expressed as mean ±SD
[a] $p < 0.01$, and [b] $p < 0.001$ versus non-diabetic control rats
[c] $p < 0.01$ versus STZ-diabetic rats

Table 2. Body weight (g) in non-diabetic, untreated STZ-diabetic and diabetic rats treated with enelapril (E), valsartan (V) or a combination of E and V (E+V)

Day	*Non-diabetic* (n = 5)	*STZ-diabetic* (n = 9)	*STZ-E* n = 9)	*STZ-V* (n = 9)	*STZ-E+V* (n = 9)
0	198.00 ± 15.76	196.33 ± 30.98	202.22 ± 16.11	198.22 ± 9.24	205.33 ± 8.51
30	199.00 ± 11.74 [a]	193.44 ± 21.01 [b]	192.00 ± 22.66 [b]	196.44 ± 14.39 [b]	179.33 ± 21.68 [b]

Values are expressed as mean ± SD
[a] $p < 0.05$, and [b] $p < 0.01$ versus day 0

Table 3. Kidney weight (KW, g) and glomerular size (GS, µm) in non-diabetic, untreated STZ-diabetic and diabetic rats treated with enelapril (E), valsartan (V) or a combination of E and V (E+V). Data are presented for day 30

Day	*Non-diabetic* (n = 5)	*STZ-diabetic* (n = 9)	*STZ-E* n = 9)	*STZ-V* (n = 9)	*STZ-E+V* (n = 9)
KW	0.80 ±0.12	0.84 ± 0.06	0.81 ± 0.20	0.89 ± 0.15	0.83 ± 0.08
GS	79.56 ±4.73	92.80 ± 5.61*	83.46 ± 2.48**	86.23 ± 4.47**	86.80 ± 4.21**

Values are expressed as means ± SD
*$p < 0.05$, versus non-diabetic control rats; **$p < 0.05$, versus STZ-diabetic rats

Table 4. Microalbuminuria levels (mg/24 hours) in non-diabetic, untreated STZ-diabetic and diabetic rats treated with enelapril (E), valsartan (V) or a combination of E and V (E+V)

Day	*Non-diabetic* (n = 5)	*STZ-diabetic* (n = 9)	*STZ-E* n = 9)	*STZ-V* (n = 9)	*STZ-E+V* (n = 9)
0	17.11 ±0.79	17.43 ± 0.84	16.75 ± 0.96	17.88 ± 0.64	17.40 ± 0.67
15	17.56 ±0.69	49.57 ± 0.20 [a]	19.25 ± 0.25	45.25 ± 1.85 [a]	18.30 ± 0.50
30	17.11 ±0.79	49.57 ± 0.20 [b]	16.75 ± 0.96 [c]	16.25 ± 0.67 [c]	17.30 ± 0.65 [c]

Values are expressed as means ± SD
[a, b] $p < 0.001$ versus non-diabetic control rats; [c] $p < 0.01$ versus STZ-diabetic rats

Table 5. Changes in immunoreactivity of TGF-b1 in glomeruli in non-diabetic, untreated STZ-diabetic and diabetic rats treated with enelapril (E), valsartan (V) or a combination of E and V (E+V)

	Non-diabetic	STZ-diabetic	STZ-V	STZ-E	STZ-E+V
TGF-β1	+	++++	++	++	++

++++, very strong immunopositivity; +++, strong immunopositivity; ++, moderate immunopositivity; +, weak immunopositivity

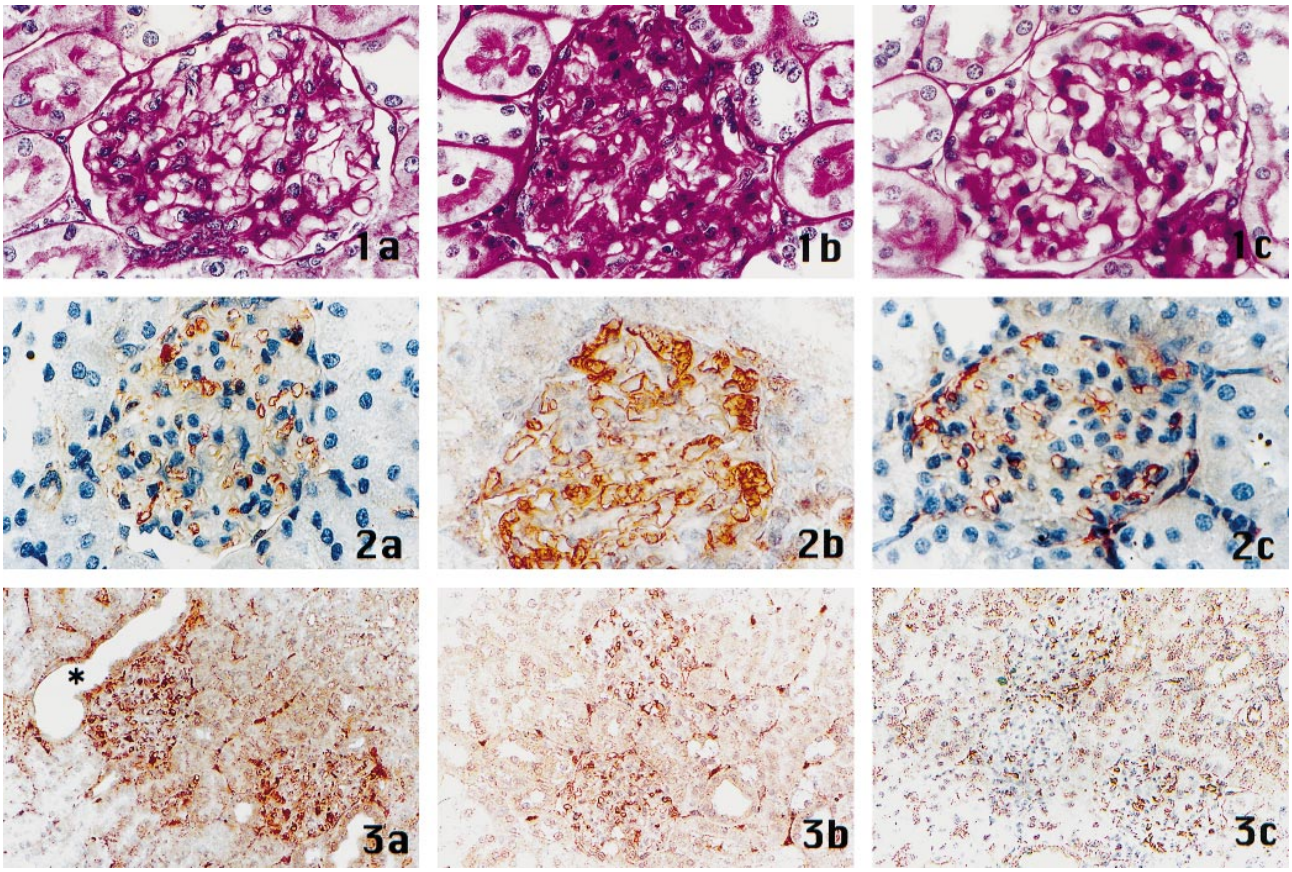

Fig. 1. PAS staining of kidney sections of non-diabetic control (**a**), untreated diabetic (**b**) and valsartan-treated diabetic (**c**) rats. Increased mesangial matrix and thickened filtration membranes are present in the glomerulus of diabetic rats as compared with the non-diabetic control rat and the valsartan-treated diabetic rat. Original magnification, × 100.

Fig. 2. Immunoperoxidase staining of TGF-β1 in glomeruli of non-diabetic control (**a**), untreated STZ-diabetic (**b**) and valsartan+enalapril-treated diabetic (**c**) rats. Increased immunostaining is present in the glomerulus of the diabetic rat as compared with the non-diabetic control rat and the treated diabetic rat. Original magnification, × 100.

Fig. 3. Immunoperoxidase staining of TGF-β1 in glomeruli of an untreated STZ-diabetic (**a**), enalapril-treated diabetic (**b**) and valsartan-treated diabetic (**c**) rats. Immunostaining is decreased in glomeruli of enalapril-treated and valsartan-treated diabetic rats as compared with untreated diabetic rats. Kidneys of untreated STZ-diabetic rats contain dilated tubules (asterix). Original magnification, × 40.

It is well known that ACE inhibitors inhibit proteinuria and delay or prevent progression of diabetic nephropathy in human and experimental diabetes (Anderson et al., 1989, 1992; Kasiske et al., 1993; Yokoyama et al, 1997). This action is independent of their downregulating effects on blood pressure (Clark and Lee, 1995). ACE inhibitors decrease AII levels and degradation of kinins but increase plasma and tissue bradykinin levels (Campbell et al., 1994). Bradykinin stimulates the production of nitric oxide and prostaglandins, and has vasodilator effects on efferent arterioles. Thus, the contribution of the kallikrein-kinin system and vasodilator prostaglandins to the renoprotective effect of ACE inhibitors via RAS inhibition remains controversial (Burns, 2000).

AT_1-receptor antagonists are orally active drugs with a specific blocking action on AT_1-receptors. In contrast with ACE inhibitors, they completely block the action of AII via AT_1-receptors. AT_1-receptor antagonists have also been shown to have renoprotective properties as ACE inhibitors do (Remuzzi et al., 1993; Kohzuki et al., 1995b). It is widely accepted that their renoprotective effects are exerted via AT_1-receptor blockade. AT_1-receptor antagonists do not affect kinin levels primarily, but increase AII levels in plasma and kidneys.

AT_1 antagonism stimulate available AT_2 receptors by AII. Experimental studies have shown that anti-proliferative and anti-fibrotic effects can be induced via AT_2 receptors in contrast with AT_1 receptor activation in the kidney. This activation of AT_2 receptors may contribute

to the beneficial properties of AT_1-receptor antagonists (Mann, 1999; Morrisey et al., 1999; Burns, 2000).

Several studies have investigated the effects of ACE inhibitors and AT_1-receptor antagonists on glucose/insulin metabolism. The matter is still very controversial. Jacob et al. (1996) have shown that ACE inhibitors can improve insulin sensitivity and this improvement is associated with favorable adaptive responses in GLUT-4 protein levels. Henriksen et al. (1999) have reported that modulation of the action of insulin by bradykinin is likely the cause of the metabolic effects of ACE inhibitors. Chow et al. (1997) have demonstrated that ACE inhibitors but not AT_1-receptor antagonists reduce the insulin that is required to dispose of a glucose load. Henriksen et all. (2001) have shown that AT_1-receptor antagonists improve glucose tolerance, at least in part because of an increase in GLUT-4 protein expression. In the present study, BG levels in the enalapril-treated or enalapril and valsartan-treated STZ-diabetic rats were significantly lower than those in the untreated STZ-diabetic rats at the end of the experiment. However, BG levels were not different in valsartan-treated rats as compared with untreated STZ-diabetic rats. These results may be related to the possible effects of ACE inhibitors on insulin sensitivity, which may not be the case for AT_1-receptor antagonists.

ACE inhibitors have been shown to decrease glomerular capillary pressure more than AT_1-receptor antagonists do and this action is inhibited by the bradykinin antagonist HO 140 (Kon et al., 1993; Komers and Cooper, 1995). A study on the effects of enalapril and losartan on albuminuria in rats with passive Heymann nephritis suggested that the benefical effects of enalapril on albuminuria are a result of decreased degradation of bradykinin rather than inhibition of AII activity (Hutchison et al., 1992). Another study showed that ACE inhibitors decrease proteinuria by increasing bradykinin levels in early phases and decreased AII production in late phases in an animal model of nephritis (Tanaka et al., 1994). However, all these studies were performed only in relatively short-term experimental models of renal impairment. In the present study, Ma levels were different in both enalapril-treated and valsartan-treated rats as compared with those in untreated STZ-diabetics. Treatment with enalapril and the combination of low doses of enalapril and valsartan resulted in Ma levels on day 15 and at end of the experiment to be similar to those in non-diabetic rats. Treatment with valsartan alone lead to Ma levels that were similar to those in non-treated diabetic rats at day 15 but returned to the levels of non-diabetic controls at the end of the experiment. These findings indicate that mechanisms of action of ACE inhibitors and AT_1-receptor antagonists on proteinuria are not the same at least in the acute phase.

Remuzzi et al. (1993) studied short-term and long-term effects of the AT_1-receptor antagonist losartan in diabetic rats. It was suggested that the renoprotective effects of AT_1-receptor antagonists are due to decreased AII activity. Lafayette et al. (1992) showed that ACE inhibitors and AT_1-receptor antagonists were equally effective in rats with reduced renal mass and the beneficiary effects of ACE inhibition was due to decreased AII activity. In the present study, there was no significant difference in morphological changes between the enalapril- and/or valsartan-treated STZ diabetic rats at the end of the experiment. There was evidence that enalapril and valsartan effectively protected against enlargement of the glomerular size as compared with STZ-diabetic rats. Although valsartan did not show similar antiproteinuric effects as compared with enalapril in the acute stage, it was as effective as enalapril in preventing glomerular changes. As a result, enalapril and valsartan had similar anti-proteinuric and renoprotective properties in our study. These findings suggest that the main mechanism of renoprotective effects of enalapril and valsartan is the blockade of action of AII mediated via AT_1-receptors.

Several studies report on the potential role of TGF-β as mediator in diabetic nephropathy (Sharma and Ziyadeh, 1995; Gilbert et al., 1998; Hill et al., 2000) and vascular injury at sites other than kidney in diabetes (Rumble et al., 1998). The stimulus triggering TGF-β expression in diabetes is hyperglycemia and/or activation of local RAS in the kidneys (Border and Noble, 1994; Wolf, 1998). It has been reported that treatment with ACE inhibitors reduced levels of TGF-β isoforms and their receptors in glomeruli of STZ-induced diabetes in rats (Hill et al., 2001). Management of glycemia by insulin treatment in STZ-induced diabetes in rats causes a decrease in expression of TGF-β in glomeruli (Fukui et al., 1992). It was found that the ACE inhibitor cilazapril and the AT_1-receptor antagonist candesartan prevent proteinuria, protect against renal tissue damage and cause a decrease in mRNA and protein levels of TGF-β in rats with progressive mesangioproliferative glomerulonephritis (Nakamura et al., 1999). It was suggested that renoprotective actions of ACE inhibitors and AT_1-receptor antagonists occur via modulation of the effects of AII on production of TGF-β and matrix proteins via AT_1 receptors. In the present study, TGF-β1 immunopositivity was found to be higher in glomeruli of STZ-diabetic rats as compared with non-diabetic rats. TGF-β1 immunopositivity was found to be weaker in glomeruli of treated diabetic rats than in untreated diabetic rats. These findings suggest that the renoprotective effect of enalapril and valsartan is associated with RAS blockade and decreased TGF-β1 synthesis.

In combination therapy, doses of the ACE inhibitor and the AT_1-receptor antagonist were reduced because such a combination has a greater effect on blood pressure (BP; Webb et al., 1998; Kim et al., 2001) although the beneficial effect on proteinuria is independent of BP levels (Clark and Lee, 1995). It was suggested that lower doses of individual agents may result in less side effects (Webb et al., 1998; Kim et al., 2001). It has been demonstrated that both ACE inhibitors and AT_1-receptor antagonists have beneficial effects in STZ-diabetic transgenic rats (Wilkinson-Berka et al., 2001) and in a rat model of diastolic heart failure (Kim et al., 2001) but their combination has stronger beneficial effects than either agent alone. In contrast, Kohzuki et al. (1995a) and Peters et al. (1998) showed that combined ACE inhibitor and AT_1-receptor antagonist therapy was not superior over therapy with these agents alone. In the present study, monotherapy and combined therapy with lower doses had similar effects on microalbuminuria, renal morphology and renal TGF-β1 immunopositivity in STZ-induced diabetes.

In conclusion, our data show that enalapril, valsartan and combination of these drugs have beneficial effects on diabetic nephropathy and their renoprotective actions may result from RAS blockade and decreased expression of TGF-β1. However, enalapril and valsartan showed to have different effects on glucose metabolism in STZ-diabetic rats. Finally, it can be concluded that combinational therapy has the advantage of effectiveness at lower doses.

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