Effects of the combination of an angiotensin II antagonist with an HMG-CoA reductase inhibitor in experimental diabetes

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Background. Angiotensin II type 1 (AT1) receptor antagonists and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have been shown to confer renoprotection. However, the renal effects of the combination of an AT1 receptor antagonist and an HMG-CoA reductase inhibitor in experimental diabetes are unknown.

Methods. Diabetes was induced by injection of streptozotocin in Wistar rats. Diabetic rats were randomly treated with losartan, an AT1 receptor antagonist, or simvastatin, an HMG-CoA reductase inhibitor, as well as the combination of both for eight weeks. Albumin excretion rate (AER) and plasma concentrations of blood urea nitrogen (BUN), creatinine, cholesterol, and triglycerides were measured. Renal injury was evaluated. Immunohistochemical staining of transforming growth factor β1 (TGFβ1) and vascular endothelial growth factor (VEGF) were performed.

Results. Increased AER in diabetic rats was attenuated by treatment with either losartan or simvastatin and further reduced by the combination of the two. Elevated plasma concentrations of BUN and creatinine were only reduced by the combination. There was no significant difference in plasma concentrations of cholesterol and triglycerides between control and diabetic rats and neither was influenced by losartan or simvastatin. Kidney pathologic injury was attenuated by losartan, but not simvastatin, compared to diabetic animals. Overexpression of TGFβ1 and VEGF was observed in the glomeruli of diabetic rats and was attenuated by losartan, simvastatin, or the combination of both to a similar level.

Conclusion. The combination of an angiotensin antagonist with an HMG-CoA reductase inhibitor confers superiority over monotherapies on renal function, as assessed by prevention of albuminuria and rise in plasma BUN and creatinine. However, no advantage of combination therapy was seen with respect to attenuating renal structural injury and renal expression of TGFβ1 and VEGF in experimental diabetes.

Key words: angiotensin II antagonist, statins, albuminuria, TGFβ1, VEGF, diabetes.

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Diabetic nephropathy is the leading cause of end-stage renal disease and a major cause of morbidity and mortality in the western world. Experimental and clinical studies over the last two decades have suggested that albuminuria, per se, may not only be a marker of renal injury but also an independent risk factor for the progression of kidney damage in diabetes. Amelioration of albuminuria is now considered one of the targets in the prevention and retardation of diabetic nephropathy.

Clinical and experimental studies have shown that blockade of the renin angiotensin system with an angiotensin II type 1 (AT1) receptor antagonist can retard the progression of kidney injury, including reducing albuminuria [1]. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (statins) have been previously demonstrated in diabetic patients the ability to reduce albuminuria [2, 3]. In experimental diabetes, fluvastatin has been shown to reduce albuminuria [4]. Furthermore, administration of statins in experimental diabetes has previously been reported to be associated with a reduction in the renal expression of the proinflammatory cytokine, transforming growth factor-β1 (TGF-β1) [5]. A recent meta-analysis provided evidence that statins reduce the rate of decline in renal function in a group of subjects with predominantly nondiabetic renal diseases [6]. There was a trend toward reduction in proteinuria or albuminuria with statins, but this did not reach statistical significance [6].

Renal protection with either an AT1 receptor antagonist or an HMG-CoA reductase inhibitor as monotherapy is suboptimal. A recent study has demonstrated that fluvastatin, an HMG-CoA reductase inhibitor, enhances the inhibitory effects of the AT1 receptor antagonist, valsartan, on vascular neointimal formation [7]. This suggests that statins may enhance the vasoprotective actions of AT1 receptor antagonist. In a rat model of passive Heymann nephritis, addition of simvastatin to the angiotensin-converting enzyme (ACE) inhibitor, lisinopril, resulted in greater reduction in proteinuria than was seen.
with lisinopril alone [8]. These data suggest that a therapy combining an ACE inhibitor with a statin may be useful in progressive renal disease. Furthermore, the renal effects of statins may be independent from their lipid-lowering effects [8]. However, there are no reports as of yet from either clinical or animal studies to determine if combining an AT1 receptor antagonist with an HMG-CoA reductase inhibitor leads to superior renoprotection in diabetes than with either agent alone.

Therefore, the aim of the present study was to assess the renal effects of a combination of an AT1 receptor antagonist with an HMG-CoA reductase inhibitor in experimental diabetes. In addition, we explored the effects of these therapies on protein expression of TGF-β1 [9, 10] and vascular endothelial growth factor (VEGF), which were considered to be important mediators of injury leading to kidney injury in diabetes [11, 12].

**METHODS**

**Animals**

Eight-week-old Wistar rats, both male and female (body weight, 220–230 g), housed at the Biological Research Laboratory in Shanxi Medical University, were used in this study. The research protocol was in accordance with the principles approved by the animal ethics committee of Shanxi Medical University. Diabetes was induced by intraperitoneal injection of streptozotocin (Boehringer-Mannheim, Mannheim, Germany) at a dose of 60 mg/kg body weight, 220–230 g), housed at the Biological Research Laboratory in Shanxi Medical University, were used in this study. The research protocol was in accordance with the principles approved by the animal ethics committee of Shanxi Medical University. Diabetes was induced by intraperitoneal injection of streptozotocin (Boehringer-Mannheim, Mannheim, Germany) at a dose of 60 mg/kg in citrate following 16-hour fasting. The animals had access ad libitum to water and standard rat chow.

**Drug therapy**

Following the induction of diabetes, the animals were randomly allocated into 4 groups with 10 rats per group (male/female = 5/5) and treated for 8 weeks. The groups are as follows: (1) diabetic rats with no treatment; (2) diabetic rats treated with the AT1 receptor antagonist, losartan, at a dose of 20 mg/kg/body weight/day by gavage (D + losartan); (3) diabetic rats treated with the HMG-CoA reductase inhibitor, simvastatin, 2 mg/kg/day by gavage (D + simvastatin); and (4) diabetic rats treated with the combination of both losartan and simvastatin at the doses described above (D + losartan + simvastatin). In addition, 10 age-matched nondiabetic rats were gavaged with the vehicle daily and served as controls (N = 10).

**Metabolic parameters and tissue collection**

Body weight was measured at the conclusion of the experiment. Prior to sacrifice, animals were placed in metabolic cages (Iffa Credo, L’Arbresele, France) for collection of urine over 24 hours for measurement of albumin concentration by radioimmunoassay (RIA) as previously described [13]. Animals were then anesthetized by intravenous injection of pentobarbital sodium (60 mg/kg/body weight). A midline incision of the abdomen was cut and blood samples were collected from the aorta for measurement of plasma concentrations of glucose, creatinine, blood urea nitrogen (BUN), total cholesterol, and triglycerides by autoanalyzer (Beckman Instruments, Palo Alto, CA, USA). Kidneys were removed and fixed in 10% formalin and processed in paraffin for subsequent histologic assessment and immunohistochemical studies.

**Kidney histopathology**

Evidence of glomerulosclerosis and tubulointerstitial injury were performed as described previously [14]. In brief, kidney sections were stained with hematoxylin and eosin and observed under a light microscope in a masked fashion at a magnification of ×400 using the Imaging Analysis System (AIS; Imaging Research, St. Catherines, Ontario, Canada) associated with a video camera and computer. All glomeruli in each kidney section (~150) were graded according to the severity of the glomerular damage: 0, normal; 1, slight glomerular damage of the mesangial matrix and/or hyalinosis with focal adhesion involving <25% of the glomerulus; 2, sclerosis of 25% to 50%; 3, sclerosis of 50% to 75%; and 4, sclerosis of >75% of the glomerulus. Tubulointerstitial area in the cortex was evaluated and graded as: 0, normal; 1, the area of interstitial inflammation and fibrosis, tubular atrophy and dilation with cast formation involving <25% of the field; 2, lesion area between 25% and 50% of the field; and 3, lesions involving >50% of the field. The indices for glomerulosclerosis and tubulointerstitial injury were calculated by averaging the grades assigned to all glomeruli and tubule fields, respectively.

**Expression of TGF-β1 and VEGF**

Expression of TGF-β1 and VEGF was assessed by using immunohistochemical techniques [15]. In brief, following dewaxing, sections were treated in a microwave oven at low power for 10 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) [16]. Endogenous peroxidase was inactivated using 3% hydrogen peroxide (H2O2) in methanol for 20 minutes, then blocked with protein blocking agent for 20 minutes. The kidney sections were incubated overnight with a rabbit polyclonal antibody to TGF-β1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or mouse monoclonal antibody to VEGF (Santa Cruz Biotechnology, Inc.). Biotinylated horse anti-rabbit or antimouse immunoglobulin (Vector Laboratories, Burlingame, CA, USA) was used as a second antibody, followed by horseradish peroxidase-conjugated streptavidin. Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, MO, USA). Quantification of TGF-β1 and VEGF immunostaining was performed by calculation of the proportion of area occupied by the
brown staining in all glomeruli or tubular area per section using the Imaging Analysis System (Imaging Research) associated with a video camera and computer as described previously [17].

Statistical analysis

Only animals that survived until the conclusion of the experiment and whose plasma glucose concentrations were greater than 15 mmol/L were included. Specifically, 44 rats survived until the end of the experiment with 6 deaths during the first week of induction of diabetes (2 deaths caused by gavage error and 4 deaths probably caused by hyperglycemia or hypoglycemia). Among the 44 surviving rats, 3 were excluded because plasma glucose levels in these rats were less than 15 mmol/L. Data were analyzed by analysis of variance (ANOVA) using Statview SE+ Graphics (Brainpower, Calabasas, CA, USA) on a Macintosh computer (iMac, Cupertino, California, USA). Comparisons of group means were performed by Fisher’s least significant difference method. Because albuminuria data did not have a normal distribution, this parameter was analyzed after logarithmic transformation. Data are shown as mean ± SEM unless otherwise specified. A P value less than 0.05 was viewed as statistically significant.

RESULTS

Metabolic parameters

Diabetic animals had reduced weight gain and increased plasma glucose concentration (Table 1). However, these parameters were not significantly influenced by any of the drug therapies. There was no significant difference in plasma total cholesterol and triglycerides between control and diabetic rats (Table 1). These parameters were not significantly influenced by any treatment.

Albuminuria

Albumin excretion rate was significantly increased in diabetic rats when compared to control rats (P < 0.01, Fig. 1A). Treatment with both losartan and simvastatin attenuated the increase in albuminuria in the diabetic rats, but this level was still higher than that observed in control rats. The combination of losartan and simvastatin was associated with a further reduction in albuminuria than was seen with either drug administrated alone, the similar albumin excretion rate (AER) level to that observed in control animals (Fig. 1A).

Plasma concentrations of BUN and creatinine

Plasma concentrations of BUN and creatinine were moderately elevated in diabetic animals (Fig. 1B and C). These two parameters were not significantly influenced by either losartan or simvastatin treatment. However, the combination of losartan and simvastatin was associated with reduction in both BUN and creatinine concentrations (Fig. 1B and C).

Kidney histology

The diabetic rats had an increase in glomerulosclerosis index when compared to control animals (Figs. 2 and 3A). This increased glomerulosclerosis index in diabetic rats was reduced by treatment with losartan. Treatment with simvastatin was associated with a trend toward lesser glomerulosclerosis index, but this did not reach statistical significance. The combination of losartan with simvastatin was associated with a reduced glomerulosclerosis index when compared to untreated diabetic rats but was not significantly different to that seen in rats receiving either monotherapy.

Diabetic rats had an increase in the tubulointerstitial injury index when compared to control rats (Fig. 3B). All treatments were associated with a trend toward less tubulointerstitial injury index, but this did not reach statistical significance.

TGF-β1 expression

TGF-β1 protein immunostaining was observed in the glomerulus and, to a lesser degree, the tubulointerstitium in control rats (Fig. 4). Immunostaining for TGF-β1 was increased in the diabetic kidney (12.1 ± 0.4% vs. control, 6.8 ± 0.4%, P < 0.01). TGF-β1 protein expression was reduced by treatment with either losartan (7.3 ± 0.5%)

| Table 1. Body weight, plasma glucose, cholesterol and triglycerides in rats after treatment with losartan, simvastatin or the combination |
|-----------------|-----------------|----------------|-----------------|-----------------|
| Group           | N               | Male/female    | Body weight g  | Glucose mmol/L | TC mmol/L      | TG mmol/L       |
| Control         | 10              | 5/5            | 261 ± 5        | 5.2 ± 0.1      | 1.56 ± 0.02    | 0.51 ± 0.01     |
| Diabetic        | 8               | 4/4            | 214 ± 6*       | 23.3 ± 0.9*    | 1.51 ± 0.05    | 0.79 ± 0.04     |
| D + Losartan   | 7               | 4/3            | 221 ± 2*       | 25.3 ± 1.0*    | 1.88 ± 0.10    | 0.65 ± 0.03     |
| D + Simvastatin| 6               | 5/1            | 221 ± 5*       | 23.9 ± 0.6*    | 1.52 ± 0.05    | 0.68 ± 0.02     |
| D + Losartan + Simvastatin | 10 | 5/5            | 210 ± 4*       | 21.8 ± 0.6*    | 1.35 ± 0.03    | 0.58 ± 0.02     |

Abbreviations are: TC, total cholesterol; TG, triglycerides. *P < 0.05 vs. control.
Fig. 1. Albumin excretion rate (A), plasma BUN (B), and creatinine (C) in control, untreated diabetic rats, and diabetic animals after 8 weeks treatment with losartan (D + losartan), simvastatin (D + simvastatin), and the combination (D + losartan + simvastatin). Albuminuria was expressed as geometric mean ± tolerance factor (mg/day). *P < 0.05 vs. control; †P < 0.05 vs. diabetic; ‡P < 0.05 vs. D + losartan and D + simvastatin. BUN is blood urea nitrogen.

or simvastatin (8.5 ± 0.4%) to levels similar to those seen in control rats. The combination of losartan and simvastatin did not result in further reduction in renal TGF-β1 expression (7.0 ± 0.5%).

**VEGF expression**

VEGF immunostaining was observed in the glomerulus and in the tubulointerstitium in control rats (Fig. 5). Immunostaining for VEGF was increased in the diabetic kidney (21.0 ± 2.2% vs. control, 9.8 ± 0.7%, P < 0.01). VEGF protein expression was reduced by treatment with either losartan (10.9 ± 0.8%) or simvastatin (12.9 ± 0.9%) to levels similar to those seen in control rats (9.8 ± 0.7%). The combination of losartan and simvastatin did not result in a further reduction in VEGF immunostaining (10.5 ± 0.9%).

**DISCUSSION**

The present study has demonstrated that the both an angiotensin II receptor antagonist and an HMG-CoA reductase inhibitor attenuate the increase in albuminuria and overexpression of TGF-β1 and VEGF in the kidney of experimental diabetes. The combination of losartan and simvastatin was associated with a further attenuation of reduction in albuminuria. Furthermore, the combination prevented the diabetes-associated increase in plasma concentrations of creatinine and urea, a marker of renal function. This phenomenon was not observed with either monotherapy. These findings provide evidence that the combination of an angiotensin II antagonist and an HMG-CoA reductase inhibitor may provide a new therapeutic approach to optimal renoprotection in diabetes.

In the present study, increased albuminuria in diabetic rats was attenuated by both losartan and simvastatin to a similar level, albeit this parameter was not normalized...
Fig. 3. Glomerulosclerosis (A) and tubulointerstitial injury indices (B) in control, untreated diabetic rats, and diabetic animals after 8 weeks treatment with losartan (D) + losartan, simvastatin (D) + simvastatin, and the combination (D) + losartan + simvastatin. *P < 0.05 vs. control; †P < 0.05 vs. diabetic.

by these monotherapies, which is still higher than that observed in control rats. These findings are consistent with our previous reports that described the reduction in albuminuria by angiotensin II antagonists in diabetic rats [14] and by HMG-CoA reductase inhibitor in a model of hemodynamic-mediated renal injury, subtotal nephrectomized rats [18]. The present study confirms that the antiproteinuric effects of HMG-CoA reductase inhibitors also occur in experimental diabetes.

The combination of losartan and simvastatin was associated with a further reduction in albuminuria than either agent alone. The mechanisms underlying this combination, leading to further reduction in albuminuria, are unknown. It is possible that the statins may directly influence the renin-angiotensin system (RAS). Recent studies have

Fig. 4. Representative photomicrographs of immunohistochemical staining of TGF-β1 in control (A), untreated diabetic rats (B), and diabetic animals treated with losartan (C), simvastatin (D), and the combination (E). TGF-β1 is transforming growth factor β1. Reproduction of this figure in color was made possible by Merck Research Laboratories.

Fig. 5. Representative photomicrographs of immunohistochemical staining of VEGF in control rats (A), untreated diabetic rats (B), and diabetic animals treated with losartan (C), simvastatin (D), and the combination (E). VEGF is vascular endothelial growth factor. Reproduction of this figure in color was made possible by Merck Research Laboratories.
suggested that statins may downregulate the AT1 receptor expression and partly mediate the angiotensin II-induced vascular function [19, 20].

The present study has confirmed an increase in TGF-β1 expression in the diabetic kidney and further demonstrated that this increase was prevented not only by treatment with the angiotensin II antagonist, losartan, but also with the HMG-CoA reductase inhibitor, simvastatin. TGF-β1 has been consistently suggested to play a key role in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis in progressive renal disease, including diabetic nephropathy [21]. Blockade of the RAS with either an ACE inhibitor or an AT1 receptor antagonist significantly reduces the expression of this prosclerotic cytokine [22]. However, the combination of losartan and simvastatin was not associated with further reduction in TGF-β1 expression. This is consistent with the view that there is not a direct correlation between blockade of the RAS and reduction in TGF-β1. Indeed, more potent blockade of the RAS by combined ACE inhibition and angiotensin II receptor antagonism is not associated with further reduction in TGF-β1 expression when compared to monotherapies [14, 23, 24].

The present study showed increased VEGF expression in the diabetic rat kidney, consistent with previous findings by several groups [25–27]. The increase in VEGF was not only attenuated by the AT1 receptor antagonist [28], but also by the HMG-CoA reductase inhibitor simvastatin. This suggests that the beneficial effects of statins include not only attenuation of TGF-β1, but also of VEGF within the kidney. Several studies have shown that statins may have other biologic effects, including regulating the expression of a range of cytokines and growth factors [29]. As demonstrated in the present study, the regulatory effects of simvastatin on TGF-β1 and VEGF occurred in the setting of no significant influence on plasma cholesterol and triglycerides. These findings support the view that statins may confer renal protection independent of their effects as lipid-lowering agents [29].

In this model of experimental diabetes, in association with increase in albuminuria, there was a modest increase in plasma creatinine and urea concentrations in diabetic animals. These parameters of renal function were only prevented by the combined treatment of losartan and simvastatin. Although not explained in the present study, this is consistent with superior renoprotection by the combination and complementary to the findings with respect to albuminuria. Indeed, a previous study has evaluated the effects of the ACE inhibitor, enalapril, and lovastatin on renal function in diabetic rats and showed a possible superiority of the combination over enalapril, but not lovastatin treatment [30].

In the present study, there was no clear-cut evidence of a superiority of combination therapy on renal structural parameters or expression of growth factors. This lack of superiority of combination over monotherapies may partly relate to the number of rats studied per group, or to the duration of diabetes only lasting eight weeks. However, in another study of eight weeks’ duration, no superiority of the combination of enalapril with lovastatin was seen on mesangial matrix expression [30]. These data are consistent with previous studies, indicating that there is not a direct correlation between functional and structural markers of diabetic nephropathy. Indeed, in studies using a neutralizing antibody to TGF-β1, despite effects on renal extracellular matrix accumulation, no effects on albuminuria were observed [9].

CONCLUSION

One must be cautious in extrapolating experimental data to the clinical context. However, AT1 receptor antagonists, although effective in retarding the progression of human diabetic nephropathy, do not confer total renoprotection. Therefore, it is important to identify additional strategies to not only retard but also to revise diabetic renal disease. In the context of known cardioprotective effects of statins, including in diabetes [31], it is now warranted to further explore whether this class of agents can be used to confer superior renal protection in diabetes.

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