Losartan and Pioglitazone Ameliorate Nephropathy in Experimental Metabolic Syndrome Rats

Xiang Kong, Dao-you Zhang, Hai-bing Wu, and Fang-xia Li

Department of Pharmacology, Third-Grade Pharmacology Laboratory of State Administration of Traditional Chinese Medicine, Wannan Medical College; No. 10, Weilu Road, Wuhu, Anhui 241002, China; and Department of Nephrology, Yijishan Hospital Affiliated to Wannan Medical College; No. 2, Zheshanxi Road, Wuhu, Anhui 241001, China.

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It is well known that metabolic syndrome (MS) is a risk factor for proteinuria and chronic kidney disease. Losartan (angiotensin II receptor blocker, ARB) and pioglitazone (peroxisome proliferator-activated receptor-γ, PPARγ agonist) have been shown to confer renoprotection. However, to date, whether or not an ARB and a PPARγ agonist have synergistic renoprotective effects remains controversial. Thus, the present study was designed to evaluate a combined treatment with losartan and pioglitazone in Sprague-Dawley rats fed with a high-fat, high-salt (HFS) diet and 20% sucrose solution for 16 weeks, an animal model of MS accompanying with renal lesions. Losartan, pioglitazone, and their combination were orally administered in the MS rats from 8 weeks to the end of this study. At 16 weeks, the MS rats showed the elevation in systolic blood pressure (SBP), urinary albumin excretion (UAE), and glomerulosclerosis (GS) score, but creatinine clearance, urinary protein excretion, and score of tubulointerstitial damage were not affected. Renal vascular endothelial growth factor (VEGF) protein level, mRNA and protein expression, which were respectively measured by enzyme-linked immunosorbent assay (ELISA), reverse transcription-polymerase chain reaction (RT-PCR), and Western blot analysis, were obviously decreased in the MS rats. Treatment with the combination of losartan and pioglitazone provided synergistic effects in reducing the SBP, UAE, and GS score when compared with monotherapy. These effects were not associated with ameliorated the downregulation of renal VEGF expression. Our data suggest that combined treatment with losartan and pioglitazone may offer additional advantages in treating MS nephropathy.

Key words metabolic syndrome; nephropathy; losartan; pioglitazone; vascular endothelial growth factor

Metabolic syndrome (MS) has been defined by the presence of a cluster of most dangerous risk factors, such as insulin resistance, hyperinsulinemia, and some combination of dyslipidemia and hypertension. Global lifestyle changes, including an increase in caloric intake and a reduction in physical activity, have sharply increased the number of patients with MS. Clinical studies reported that MS also increases the risk for proteinuria and chronic kidney disease independent of diabetes and hypertension. However, the underlying mechanisms and efficient therapy of nephropathy associated with MS are not completely elucidated.

Chronic consumption of high-fat, high-sucrose diet induces a MS model in normal rats, which is associated with dyslipidemia, hypertension, obesity, and insulin resistance. However, damages of renal structure and function have not been reported in this MS model. In fact, whether high-fat or high-fat, high-sucrose diet produced renal functional and pathological lesions is still conflicting. Nevertheless, several studies have demonstrated that high salt diet induces slightly hyperinsulinemia, moderately hypertension and renal damages in Sprague-Dawley (SD) rats. Therefore, we hypothesized that long-term consumption of a high-fat, high-salt diet and sucrose solution in SD rats could induce a suitable animal model that mimics the basic characteristics of human MS and is helpful to understand the pathogenesis and therapy of nephropathy associated with MS.

Clinical and experimental studies have shown that losartan (angiotensin II receptor blocker, ARB) and pioglitazone (peroxisome proliferator-activated receptor-γ, PPARγ agonist) have renoprotective effects in diabetic nephropathy (DN) and non-diabetic renal diseases. In addition, a recent study demonstrated that losartan and pioglitazone have synergistic renoprotective actions in patients with type 2 DN. However, to our knowledge, there was no study on comparing the effects of losartan and pioglitazone combination therapy with monotherapy in MS nephropathy rats.

Vascular endothelial growth factor (VEGF) is an important growth factor involved in neoangiogenesis and vascular trophism. The dysregulation of VEGF signaling system has been identified in a wide variety of renal diseases, such as DN, glomerulonephritis, acute renal failure, chronic renal disease, and so on. Qin et al. suggested that losartan attenuates the overexpression of VEGF in DN rats. Lee et al. demonstrated that pioglitazone has beneficial effects on DN in Otsuka-Long-Evans-Tokushima-Fatty rats by reducing the VEGF expression. Thus, we speculated that both drugs might improve renal damage in this MS model through attenuating dysregulation of the VEGF expression.

Therefore, the purposes of this study were: first, to establish a suitable animal model of nephropathy associated with MS; second, to evaluate the possible benefits of losartan and pioglitazone in this experimental MS nephropathy rats; third, to assess whether the combination therapy was superior to treatment with either drug alone; finally, to examine the role of VEGF express in losartan and pioglitazone-mediated renoprotection.

MATERIALS AND METHODS

Drugs and Reagents Losartan was provided by MSD Pharmaceutical Co. (Hangzhou, China). Pioglitazone was purchased from Takeda Pharmaceutical Co. (Tianjin, China).
Rat VEGF enzyme-linked immunosorbent assay (ELISA) kit, radio immunoprecipitation assay (RIPA) lysis buffer, bovine serum albumin (BSA), nitrocellulose membranes, mouse monoclonal β-actin antibody were purchased from Boster Biotechnology Inc. (Wuhan, China). Rabbit polyclonal VEGF antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) Albumin and insulin radioimmunoassay (RIA) kits were purchased from North Institute of Biotechnology (Beijing, China). Glucose, total cholesterol, and triglycerides assay kits were purchased from Rongsheng Biotechnology Co. (Shanghai, China). Total protein, urinary protein and creatinine assay kits were purchased from Jiancheng Institute of Biotechnology (Nanjing, China).

**Animals, Diet and Experimental Design** Male SD rats [Certificate No: SCXK(zhe) 20080033] aged 7—8 weeks (weighing 240±20 g) were obtained from Zhejiang Province Experimental Animal Center (Hangzhou, China). The rats were housed in individual cages at 24—26 °C with a 12-h light–dark cycle, and acclimated to these conditions for 1 week. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996). Rat chow was purchased from the Qingshong Experimetal Animal Center (Nanjing, China). High-fat, high-salt (HFS) diet was prepared as described in our previous studies with slight modification. The diet components are listed in Table 1.

After the acclimatization period, seven rats (control group) received the normal diet and water, other rats were fed with the HFS diet and 20% sucrose solution ad libitum constant to the end of this experiment, namely lasted 16 weeks. After 8 weeks received the HFS diet and sucrose solution, the animals except the control group were randomly assigned to four groups, namely metabolic syndrome (MS) model group (n=7), losartan treatment group (20 mg/kg, n=6), pioglitazone treatment group (10 mg/kg, n=6), and combined treatment group (received both losartan and pioglitazone at the doses described above, n=6). The dosages of losartan and pioglitazone were based on previous studies. The drugs were orally administrated at 9:00—10:00 a.m. every day by gavage for 8 weeks. Untreated groups received an equal volume of distilled water. During the entire period of the experiment, body weight (BW), food consumption, and water intake were measured weekly. The rats were placed in metabolic cages to collect 24 h urine at the start, 8 weeks and end of this study. Systolic blood pressure (SBP) was measured monthly in conscious rats using the tail-cuff method (ALC-NIBP, Shanghai, China) as described in our previous studies.

Two days before sacrifice, glucose tolerance was tested. At 16 weeks, the rats were fasted overnight and anesthetized by an intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg). Blood samples were drawn from abdominal aorta, centrifuged to obtain serum. Kidneys were removed, decapsulated and immediately weighed. Visceral fat mass (mesenteric, epididymal and retroperitoneal adipose tissue) was excised and weighed. Kidney weight/body weight (KW/BW) ratio and visceral fat weight/body weight (VisF/BW) ratio were calculated. One kidney (the right one) was frozen at −80 °C until processed, the other was removed for fixation in 10% formaldehyde for 24 h before process for histology.

**Determination of Glucose Tolerance** An oral glucose tolerance test (OGTT) was used to assess glucose tolerance. After 12 h fasting, tail blood samples were taken before (time 0) and 15, 30, 60, and 120 min after oral administration of a solution of 20% glucose (2 g/kg). Glucose levels were measured using a glucometer (One Touch Horizon, Life Scan Inc., U.S.A.). Animals were not anesthetized for this procedure.

**Biochemical, Radioimmunoassay, and ELISA Analysis** Levels of glucose, creatinine (Cr), total cholesterol, and triglycerides in serum and concentrations of protein and Cr in urine were determined by a biochemistry analyzer (Type-811, Shanghai, China) using commercial kits mentioned above. Serum insulin and urine albumin levels were measured using a Gamma-counter (GC-911, Hefei, China) with RIA kits. The sodium content in the urine was determined by an electrode method. Homeostatic model assessment of insulin resistance (HOMA-IR) and creatinine clearance (Ccr) were calculated using the following equations: HOMA-IR=[[fasting glucose (mmol/l)×fasting insulin (μIU/ml)/22.5], and Ccr (ml/min/kg body weight)=[[urine Cr (μmol/l)×urine volume (ml)/serum Cr (μmol/l)]×[1000/blood weight (g)]×[1/1440 (min)].

Renal tissue were homogenized in RIPA lysis buffer (containing 1% protease inhibitor cocktail) and centrifuged (12000 g at 4 °C for 15 min). The supernatant was used for ELISA and western blot analysis. Serum and renal VEGF levels were measured using a commercially ELISA kit according to the manufacturer’s instructions. Briefly, the samples were transferred to 96-well microplates. The plate was incubated at 37 °C for 1.5 h. Then, biotinylated anti-rat VEGF antibody was added to each well and incubated at 37 °C for 1 h. After three washes, avidin–biotin–peroxidase complex was added and further incubated for 30 min. After washing further five times, TMB was added to each well and absorbency at 450 nm was measured using a microplate reader (ELX 800, BioTek, U.S.A.). Values of each sample were normalized with the protein concentrations measured using Bradford assay.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis** Total RNA was extracted from the rat kidney using Trizol reagent (Gibco, U.S.A.) and converted to cDNA. The primer sequence of VEGF was selected accord-

| Table 1. Compositions of Normal and High-Fat, High-Salt (HFS) Diets |
| --- | --- | --- |
| Composition | Normal diet | HFS diet |
| Energy as protein (%) | 27.8 | 21.3 |
| Energy as carbohydrate (%) | 59.3 | 45.4 |
| Energy as fat (%) | 12.8 | 33.3 |
| Caloric density (kcal/g) | 21.7 | 21.1 |
| Protein (g/100 g) | 46.2 | 33.0 |
| Carbohydrate (g/100 g) | 2.4 | 2.4 |
| Fat (g/100 g) | 8.0 | 6.5 |
| Cellulose (g/100 g) | 0.4 | 4.0 |
| NaCl (g/100 g) | 2.4 | 0.2 |
| Mineral mix (g/100 g) | 0.2 | 0.2 |

Major protein is obtained from corn, wheat, wheat bran, soya bean, fish meal, soybean flour, and yeast powder. Fat in the HFS diet is supplied in the form of lard oil (15%) and cholesterol (1%).
and tubulointerstitial injury was evaluated by a semi-quantitative protocol. The degree of glomerulosclerosis (GS) was quantified with periodic acid Schiff (PAS) and Masson-trichrome, and the mean scores were calculated.

Statistical Analysis Data were expressed as mean ± S.E.M. For statistical analysis, we used one-way analysis of variance (ANOVA) followed by Newman–Keuls tests. p<0.05 was considered statistically significant.

RESULTS

Physiological Parameters As shown in Table 2, at 16 weeks after receiving the HFS diet and 20% sucrose solution, the BW, water intake, VisF/BW, and KW, except for the food consumption and KW/BW ratio in the MS model group were significantly higher than those in the control group (p<0.05). Compared with the model group, the VisF/BW ratio was only reduced by treatment with pioglitazone (p<0.05), and the KW was decreased by treatment with losartan and the combination of losartan and pioglitazone (p<0.05). Other parameters were not significantly influenced by any of the drug therapies.

As shown in Fig. 1A, at the beginning (0 week) of the study, no significant difference in basal level of SBP was observed among all experimental groups. At 8 weeks, the SBP in rats fed with the HFS diet and 20% sucrose solution was moderately increased compared with that in the control rats (p<0.05). At 16 weeks, the SBP in the losartan, pioglitazone, and combined treatment group (113±7, 123±5, and 102±4 mmHg, respectively) was significantly lower than that in the model group (148±8 mmHg, p<0.05). Treatment with the combination had a further reduction in the SBP than administration of either drug alone (p<0.05).

Biochemical Parameters As shown in Table 3, Figs. 1B and D, at 16 weeks, the serum levels of insulin, total cholesterol, and triglycerides, HOMA-IR, sodium excretion, and area under curve (AUC) of OGTT (area under the OGTT curve) in the model group were significantly increased compared with those in the control group (p<0.05). Treatment with pioglitazone and the combination of losartan and pioglitazone for 8 weeks obviously decreased the insulin, total cholesterol and triglycerides levels, HOMA-IR, and AUC of OGTT (p<0.05 versus model group). There were no significant differences in the glucose concentration, Ccr, sodium excretion and urinary protein excretion (UPE) among all experimental groups.

As shown in Fig. 1C, no significant difference in basal

<table>
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<th>Parameters</th>
<th>Control</th>
<th>Model</th>
<th>Losartan</th>
<th>Pioglitazone</th>
<th>Losartan + Pioglitazone</th>
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<tr>
<td>BW (g)</td>
<td>522±3.2</td>
<td>622.7±3.1 *</td>
<td>639.2±6.2 *</td>
<td>648±28.5 *</td>
<td>634±24.3 *</td>
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<tr>
<td>Food consumption (g/d)</td>
<td>24.4±1.2</td>
<td>21.2±1.7 *</td>
<td>21.4±2.5 *</td>
<td>20.3±3.1 *</td>
<td>21.9±1.7 *</td>
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<td>Water intake (ml/d)</td>
<td>37.5±5.9</td>
<td>70.8±9.5 *</td>
<td>72.6±6.2 *</td>
<td>72.8±3.1 *</td>
<td>68.8±8.9 *</td>
</tr>
<tr>
<td>VisF/BW (mg/g)</td>
<td>30.5±5.1</td>
<td>66.4±7.5 *</td>
<td>68.5±7.9 *</td>
<td>56.2±5.1 *</td>
<td>61.2±4.5 *</td>
</tr>
<tr>
<td>KW (g)</td>
<td>2.33±0.18</td>
<td>2.92±0.19 *</td>
<td>2.57±0.15 *</td>
<td>2.77±0.12 *</td>
<td>2.62±0.21 *</td>
</tr>
<tr>
<td>KW/BW (mg/g)</td>
<td>4.62±0.36</td>
<td>4.80±0.45</td>
<td>4.20±0.46</td>
<td>4.46±0.30</td>
<td>4.30±0.50</td>
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</table>

Body weight (BW), food consumption, and water intake were measured weekly during the entire period of the experiment. Values are expressed as mean±S.E.M. n=6—7 per group. †p<0.05 vs. control group. *p<0.05 vs. model group. Los: losartan (daily dose of 20 mg/kg), Pio: pioglitazone (daily dose of 10 mg/kg), Los+Pio: losartan (20 mg/kg) and pioglitazone (10 mg/kg), VisF: visceral fat, KW: kidney weight.
level of urinary albumin excretion (UAE) was recorded before the study. However, the MS model group showed a significant elevation in UAE in 8 weeks (27.3±4.7 μg/d) and 16 weeks (38.6±6.0 μg/d) on the HFS diet and 20% sucrose solution compared with that seen in the control group (12.3±2.8 and 12.8±3.1 μg/d, p<0.05). Treatment with losartan, pioglitazone, and their combination attenuated the increase in UAE (28.2±4.5, 27.2±4.8, and 21.2±4.0 μg/d, p<0.05 versus model group), but the level was still higher than that in the control group (p<0.05). The combination therapy was associated with less UAE than monotherapy (p<0.05).

Protein Level of VEGF in Serum and Kidney As shown in Fig. 2B, ELISA indicated that renal protein level of VEGF was obviously decreased in the model group (17.4±4.2 ng/mg protein) compared with the control group (24.1±4.0 ng/mg protein, p<0.05). However, no significant change in renal VEGF protein level was observed among all treatment groups, individually and in combination (20.6±2.8, 22.2±3.0, and 20.7±3.4 ng/mg protein) compared with the model group. As shown in Fig. 2A, the serum level of VEGF protein was not significantly different among all experimental groups.

VEGF mRNA and Protein Expression in Kidney RT-PCR (Fig. 2C) and Western blot analysis, respectively. Panels show representative bands and histograms represent optical density values normalized to the corresponding β-actin. n=4–5 per group, p<0.05 vs. control group.

Renal Histopathology As illustrated in Fig. 3, the MS rats displayed mild lesions of the glomeruli such as slight tuft adhesions and segmental sclerosis, with a GS score of
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1.02±0.26 versus 0.16±0.05 in the control rats (p<0.05). The GS score was significantly lower in the losartan (0.66±0.10), pioglitazone (0.70±0.11) and combined treatment group (0.48±0.09) compared with the model group (p<0.05), the combination of both drugs superior to either alone (p<0.05). In addition, the MS rats exhibited no obviously tubulointerstitial damage (no tubular atrophy and interstitial fibrosis, and less than 1% of inflammation). There was no significant difference in the tubulointerstitial injury score among all experimental groups.

DISCUSSION

The major conclusions to be drawn from this study were: 1) SD rats fed with the HFS diet and 20% sucrose solution for 16 weeks could induced a suitable MS animal model associated with nephropathy, such as albuminuria and glomerulosclerosis; 2) treatment with the combination of losartan and pioglitazone provided synergistic effects in reducing SBP, UAE, and GS score when compared with monotherapy; and 3) the renal VEGF protein level, mRNA and protein expression were decreased in the MS rats, whereas not affected by both drugs, individually and in combination.

In the present study, at 8 weeks after received the HFS diet and 20% sucrose solution, rats showed a significant elevation in the albuminuria and GS score (p<0.05). In addition, the MS rats exhibited no obviously tubulointerstitial damage (no tubular atrophy and interstitial fibrosis, and less than 1% of inflammation). There was no significant difference in the tubulointerstitial injury score among all experimental groups.

The accumulation of VisF is the most important cause of MS, and excess adipose tissue reduces insulin sensitivity in metabolically responsive tissues. Consistent with a recent study, we demonstrated that pioglitazone significantly suppressed the increases in VisF accumulation in the MS rats. In our experimental conditions, we also found improvement in dyslipidemia and insulin resistance after treatment with pioglitazone, and these were the main causes contributed to the suppression of VisF/BW ratio. In addition, the similar effects were observed after the combination therapy. Taken together, the combination of losartan plus pioglitazone is equally effective compared to pioglitazone, but than losartan single applications concerning hypolipidemic effect and amelioration of insulin resistance.

Long-term administration of losartan, pioglitazone, and their combination reduced the UAE and GS score in the MS model rats. These results confirmed previous evidences that both losartan and pioglitazone have renoprotective effects. In addition, the renoprotection accompanying with obvious antihypertensive action was observed in all treatment groups. The combination therapy had a further antihypertensive effect than monotherapy, accompanied by a fall in albuminuria and GS score. Our study did not exclude that reduction in blood pressure per se and other effects might contribute to the renoprotective effects of losartan and pioglitazone. However, several studies have reported that reduction of blood pressure by hydralazine produces only a minimal beneficial renal effect in hypertensive Dahl salt-sensitive rats. In normotensive streptozotocin-induced diabetic nephropathy rats, losartan and pioglitazone also ameliorate damages of renal
function and histology through their pleiotropic actions, such as antioxidative and/or anti-inflammatory effects. Thus, we considered that the renoprotective effect seen in this study was partially blood pressure-dependent.

VEGF exerts a very important and unique role in maintaining renal structure and functions. Cancer patients treated with anti-VEGF therapy develop hypertension and renal damage such as proteinuria. Nevertheless, the role of VEGF in renal disease is controversial. There is a loss of VEGF in glomerulonephritis, acute renal failure, and chronic renal disease but an overabundance in DN. In our study, at 16 weeks, we found lower VEGF protein level, mRNA and protein expression in kidney from the MS model group compared with the control group. Recent studies demonstrated that both glucose and ANG II increase renal VEGF expression. However, the serum level of glucose was not affected in our experimental conditions and thereby not contributed to the alteration of VEGF expression. ANG II is a powerful sodium-retaining hormone and vasoconstrictor that is decreased by high dietary sodium intake. Gu et al. reported that a long-term high salt diet decreases renal expression of VEGF mRNA and protein in SD rats. Taken together, downregulation of ANG II level induced by high sodium intake might contribute to the reduction of renal expression of VEGF in this animal model. Nevertheless, in contrast to our prediction, treatment with losartan, pioglitazone, and their combination for 8 weeks failed to enhance renal VEGF protein level, mRNA and protein expression. These results suggested that the renoprotective effect of both drugs in this MS model was due to other causes, such as repression of VEGF in this animal model. Nevertheless, in vitro experiments demonstrated that in human proximal tubular epithelial cells activated by angiotensin II type 1 receptor (AT 1R) demonstrated that in human proximal tubular epithelial cells activated by angiotensin II type 1 receptor (AT 1R) signal-regulated kinase (ERK)1/2 activation. Nevertheless, the precise mechanisms whereby the additional renoprotection provided by the combination of a PPARγ agonist and an ARB were exerted in vivo required further investigations.

In summary, our results suggest that long-term orally administered of the combination of losartan and pioglitazone offers additional potential advantages compared with monotherapy in reducing the elevated blood pressure and improving the lesions of renal structure and function in the HFS diet and sucrose solution induced MS nephropathy rats. Thus, it is likely that combined treatment with an ARB plus a PPARγ agonist is beneficial in retarding the development of renal damage in patients with MS. However, given the limitation of animal model, whether this therapeutic strategy also works in humans requires further clinical investigations.

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REFERENCES
30) Nickern G., Streithoefl., Roeling J., Zolk O., Knorr A., Bohn M., Hy-