Effects of pioglitazone and candesartan on renal fibrosis and the intrarenal plasmin cascade in spontaneously hypercholesterolemic rats

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Effects of pioglitazone and candesartan on renal fibrosis and the intrarenal plasmin cascade in spontaneously hypercholesterolemic rats. Am J Physiol Renal Physiol 293:F1292–F1298, 2007. First published August 1, 2007; doi:10.1152/ajprenal.00232.2007.—The profibrotic effect of plasminogen activator inhibitor-1 (PAI-1) in renal fibrosis is widely recognized, but its mechanism remains controversial especially in chronic progressive kidney disease. In the present study, pioglitazone (Pio) and candesartan (CD), which are reported to inhibit PAI-1, were administered to spontaneously hypercholesterolemic (SHC) rats, a model of chronic progressive kidney disease. Therapeutic effects and effects on the intrarenal plasmin cascade were examined. Eight-wk-old SHC rats were used as controls. Oral administration of vehicle alone, Pio, or CD was performed starting at 8 wk of age and was continued for 24 wk. The degree of renal fibrosis was evaluated by sirius red staining of kidney sections and by total collagen assay of renal homogenates. The renal PAI-1 protein level was assessed by Western blotting, and plasmin activity was analyzed by chromogenic assay and casein gel zymography. Urinary protein and blood urea nitrogen were significantly increased in the vehicle-treated group, but the increase was attenuated in the Pio- and CD-treated groups. This correlated well with the degree of fibrosis as assessed by sirius red staining and total collagen assay. The PAI-1 protein level was also increased significantly in the vehicle-treated group, and the increase was attenuated in the Pio- and CD-treated groups. Despite the presumed plasmin-inhibitory function of PAI-1, plasmin activity changed in parallel with PAI-1. These results suggest that Pio and CD inhibit PAI-1 and exert renoprotective effects against chronic progressive renal disease, but its action is independent of the regulatory function on plasmin activity.

angiotensin II type 1 receptor blocker; peroxisome proliferator-activated receptor-γ agonist; plasminogen activator inhibitor-1

THE SPONTANEOUSLY HYPERCHOLESTEROLEMIC (SHC) or Imai rat was developed as a model of spontaneous hyperlipidemia on the genetic background of the Sprague-Dawley rat (8). In male SHC rats, proteinuria and hyperlipidemia develop by 8 wk of age, resulting in decreased kidney function with histological changes of focal segmental glomerulosclerosis 8–9 mo later (32). Thus the SHC rat is a good model of chronic progressive kidney disease with continuous severe proteinuria.

Many experimental and clinical studies have shown renoprotective effects of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 (AT1) receptor blockers (ARBs) (12, 27, 30, 31). Beside antihypertensive action, ACE and ARB are suspected to exhibit a renoprotective effect through an inhibitory function on plasminogen activator inhibitor-1 (PAI-1) (6, 9, 14, 16, 19). Recent studies have shown a renoprotective effect of peroxisome proliferator-activated receptor-γ (PPARγ) agonists on diabetic as well as nondiabetic renal disease (15, 28). PPARγ agonists have also been shown to decrease PAI-1 activity in mice (17).

PAI-1, which is a major physiological inhibitor of plasminogen activators, is a multifunctional protein (3, 4, 20, 34). PAI-1 also influences cell migration, independently of its mechanism to inhibit plasminogen activation (4). It remains controversial which pathways in PAI-1 are involved in the renoprotective effect. There are several studies indicating important roles of PAI-1, independent of plasmin regulation, in an acute, aggressive renal fibrosis model (5, 20, 23). Meanwhile, the detailed mechanism in a chronic progressive renal fibrosis model has not been fully elucidated.

The purpose of the present study was to examine the therapeutic effects of the ARB candesartan (CD) and the PPARγ agonist pioglitazone (Pio) as long-term therapies against renal damage in the SHC rat and the effects of these drugs on PAI-1 and the intrarenal plasmin cascade.

MATERIALS AND METHODS

Animal model. Breeding pairs of SHC rats were obtained from Takeda Chemical Industries (Osaka, Japan). Rats were given free access to tap water and standard rat chow. Animal care followed the guidelines of the National Defense Medical College (Saitama, Japan) for the care and use of laboratory animals in research. The study protocols were approved by the Animal Ethical Committee of the National Defense Medical College.

Baseline studies were initiated in 8-wk-old male SHC rats. Oral administration of drugs to 8-wk-old male SHC rats were performed continuously for 24 wk until all animals were euthanized. Rats were randomly assigned to the following groups (n = 5 each): 1) the control group (before the development of renal manifestation); 2) the vehicle-treated group, in which rats were treated with vehicle alone for 24 wk; 3) the Pio-treated group, in which rats were treated with Pio (5 mg·kg−1·day−1, Takeda Chemical Industries) for 24 wk; and 4) the CD-treated group, in which rats were treated with CD (1 mg·kg−1·day−1, Takeda Chemical Industries) for 24 wk.

Systolic blood pressure was measured by tail-cuff plethysmography at 8, 16, 24, and 32 wk of age. Urine samples were collected for 24 h in metabolic cages just before each animal was killed. Rats were killed by exsanguination under general anesthesia with diethyl ether. Serum creatinine, blood urea nitrogen, total cholesterol, triglyceride, and urinary protein levels were measured by standard methods using an automatic analyzer (Hitachi 7170, Hitachi, Tokyo, Japan). Kidneys

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were harvested, weighed, and processed for the assays described below.

**Determining degree of renal fibrosis.** Renal fibrosis level was evaluated histologically by sirius red staining and biochemically by total collagen assay. Sirius red staining was performed on paraffin sections of renal tissues as described previously (23), and stained sections were observed by polarized light microscopy.

For the total collagen assay, hydroxyproline concentration in hydrolysates of precisely weighed frozen kidney samples was measured chemically as described previously (23). Total collagen was assumed to contain 12.7% hydroxyproline by weight. Results are expressed as micrograms collagen per milligram kidney weight.

**Western blot analysis of PAI-1.** Pieces of frozen kidney were homogenized with a DIAx 100 homogenizer (Heidelberg Instruments, Schwabach, Germany) in 50 mM Tris·HCl, 0.2% Triton X-100, 50 mM NaCl, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) at pH 7.7. Protein concentrations were measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Protein samples (60 μg) were separated by 10% SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose membranes. Membranes were probed with rabbit anti-mouse PAI-1 IgG (Molecular Innovations, Southfield, MI) as the primary antibody and horse-radish peroxidase-conjugated goat anti-rabbit IgG (American Qualex International, San Clemente, CA) as the secondary antibody. Bands were detected with an enhanced chemiluminescence (ECL Plus) kit (GE Healthcare UK, Buckinghamshire, UK). Equal loading and transfer of samples were confirmed and adjusted according to the β-actin signal, which was detected with a hors eradish peroxidase-conjugated anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The density of each band was quantified with CS Analyzer, version 2.0 software (Atto, Tokyo, Japan).

**Chromogenic assay for plasmin activity.** Samples were homogenized in 50 mM Tris·HCl, 0.2% Triton X-100, 50 mM NaCl, and 3 mM EDTA, pH 7.7. Total kidney plasmin activity was measured with a plasmin-specific chromogenic substrate, Chromozym PL (Boehringer Mannheim, Indianapolis, IN), as described previously (23). Samples (10 μl) containing 170 μg kidney protein, 35 μl 50 mM Tris·HCl (pH 8.2), 5 μl 0.9% NaCl, and 10 μl chromogenic substrate were mixed in a 96-well microtiter plate. Samples were measured for absorbance at 405 nm twice over a 1-h period with a 96-well plate reader. A standard regression curve was constructed with serial dilutions of plasmin (Wako Pure Chemical Industries, Osaka, Japan).

**Casein gel zymography for plasmin activity.** Renal plasmin activity was further determined by gel zymography essentially as described previously (23). In brief, kidney protein samples (60 μg protein) isolated for Western blotting were separated under nonreducing conditions on 10% SDS polyacrylamide gels containing casein (2 mg/ml). After a wash in 2.5% Triton X-100, gels were incubated with substrate buffer for 36 h and stained with 0.002% Coomassie blue. The density of each lytic band was quantified with CS Analyzer version 2.0 software (Atto).

**In situ zymography for plasmin activity.** Plasmin activity in renal tissues was examined by in situ zymography essentially as described previously (24) but with one modification. We applied D-Val-Leu-Lys α-naphthyl ester (Torii Pharmaceutical, Tokyo, Japan) instead of Tos-Lys α-naphthyl ester as the substrate for plasmin because the former is known to be more specific for plasmin than the latter.

**Histological examination.** Interstitial macrophage infiltration was evaluated by immunoperoxidase staining for macrophages (mouse anti-rat macrophage ED1 monoclonal antibody; Chemicon International, Temecula, CA). ED1 was detected with the use of peroxidase-conjugated, rat plasma-absorbed F(ab’2) goat anti-mouse IgG (Nichirei, Tokyo, Japan) as the secondary antibody and 3,3’diaminobenzidine as the chromogen (Dako). Sections were counterstained with methyl green. Five random, nonoverlapping cortical fields (×200 magnification) for each section were assessed, and the number of ED1-positive interstitial cells in each area was counted and averaged in each group.

**Statistical analysis.** Data are shown as means ± SE. Comparisons between groups were performed by one-way ANOVA. Post hoc tests were performed with Fisher’s protected least significant difference test. P < 0.05 was considered statistically significant. Statistical analysis was performed with StatView version 5.0 software (SAS Institute, Cary, NC).

**RESULTS**

**Physiological index.** Body and kidney weights, systolic blood pressure, and serum and urine parameters are listed in Table 1. Body weight and kidney weight increased with age. The treatment groups showed increased body weight compared with that in the vehicle-treated group. There was no significant difference in kidney weight between the 32-wk-old groups. Systolic blood pressure increased with age and decreased significantly in response to treatment with Pio or CD. Systolic blood pressure was also measured at 16 and 24 wk of age (data not shown); systolic blood pressure was low at 16 wk but high at 24 wk in the vehicle-treated group (16 wk: 144.2 ± 1.7 mmHg, 24 wk: 169.6 ± 9.5 mmHg). Pio or CD inhibited this increase (16 wk: 146.1 ± 4.0 mmHg, 24 wk: 157.2 ± 8.0 mmHg in the Pio-treated group; 16 wk: 127.9 ± 1.4 mmHg, 24 wk: 130.1 ± 8.4 mmHg in the CD-treated group). Serum creatinine, blood urea nitrogen, total cholesterol, triglyceride, and urinary protein levels were significantly increased in the vehicle-treated group compared with those in the control group. The triglyceride level was significantly decreased in the Pio- and CD-treated groups compared with that in the vehicle-treated group. Urinary protein, total cholesterol, and blood urea nitrogen levels were significantly decreased in the Pio-treated group, although the CD-treated group also showed lower values than those in the vehicle-treated group. There was no significant difference in serum creatinine levels between the 32-wk-old groups.

![Table 1. Weights, systolic blood pressure, and serum and urinary markers per group of SHC rats](http://ajprenal.physiology.org/)

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>Vehicle (n = 5)</th>
<th>Pio (n = 5)</th>
<th>CD (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>293.8 ± 4.1</td>
<td>546.9 ± 13.3*</td>
<td>565.6 ± 18.2</td>
<td>624.3 ± 16.5*</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>1.17 ± 0.05</td>
<td>1.60 ± 0.10*</td>
<td>1.54 ± 0.08</td>
<td>1.70 ± 0.13</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>148.9 ± 3.6</td>
<td>173.5 ± 6.5*</td>
<td>129.6 ± 5.6†</td>
<td>116.7 ± 3.8†</td>
</tr>
<tr>
<td>Urinary protein, mg/day</td>
<td>5.0 ± 1.4</td>
<td>167.0 ± 53.3*</td>
<td>57.4 ± 15.8†</td>
<td>104.5 ± 36.2</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>13.2 ± 1.6</td>
<td>139.2 ± 26.2*</td>
<td>52.0 ± 9.6†</td>
<td>119.2 ± 11.6†</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>91.2 ± 2.9</td>
<td>216.8 ± 32.6*</td>
<td>128.4 ± 7.7†</td>
<td>188.8 ± 29.1</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>12.2 ± 0.6</td>
<td>21.7 ± 2.8*</td>
<td>15.8 ± 0.6†</td>
<td>17.8 ± 0.5</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.21 ± 0.02</td>
<td>0.42 ± 0.02*</td>
<td>0.39 ± 0.01</td>
<td>0.38 ± 0.02*</td>
</tr>
</tbody>
</table>

Values are means ± SE. SHC, spontaneously hypercholesterolemic; Pio, pioglitazone; CD, candesartan. *P < 0.01 vs. control. †P < 0.01 vs. vehicle.
The total collagen content in each group is shown in Fig. 1A. The total collagen content was significantly increased in the vehicle-treated group compared with that in the control group (6.9 ± 0.2 vs. 4.2 ± 0.1 μg/mg kidney wt, respectively; P < 0.01). Among the 32-wk-old groups, renal interstitial fibrosis was significantly improved in both the Pio- and CD-treated groups compared with that in the vehicle-treated group (Pio-treated group: 6.4 ± 0.1 μg/mg kidney protein, P < 0.01; CD-treated group: 6.1 ± 0.1 μg/mg kidney protein, P < 0.01 vs. the vehicle-treated group).

**PAI-1 protein level.** Representative Western blot results for PAI-1 and β-actin and calculated results for the PAI-1/β-actin protein ratio are shown in Fig. 2. The PAI-1/β-actin protein ratio was significantly increased in the vehicle-treated group compared with that in the control group (13.5 ± 3.4 vs. 1.0 ± 0.3, respectively; P < 0.01) and was significantly decreased in both the Pio- and CD-treated groups compared with that in the vehicle-treated group (Pio-treated group: 5.5 ± 1.2, P < 0.05; CD-treated group: 6.1 ± 1.4, P < 0.05 vs. the vehicle-treated group) (Fig. 2B).

**Plasmin activity.** Chromogenic assay results for plasmin activity are shown in Fig. 3. Plasmin activity was significantly increased in the vehicle-treated group compared with that in the control group (6.6 ± 0.8 vs. 0.6 ± 0.3 μU/ml, respectively; P < 0.01), and the activity was decreased in the Pio- and CD-treated groups compared with that in the vehicle-treated group.

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**Fig. 1. Degrees of renal fibrosis.** A: representative images of sirius red staining of renal tissue from 8-wk-old spontaneously hypercholesterolemic (SHC) rats in the control group (a), vehicle-treated group (b), pioglitazone (Pio)-treated group (c), and candesartan (CD)-treated group (d). Increased interstitial fibrosis was observed in the vehicle-treated group compared with that in control group. Interstitial fibrosis was attenuated in the Pio- and CD-treated groups compared with that in the vehicle-treated group. Magnification, ×200. B: quantitation of total collagen content. Total collagen content in the kidney was significantly increased in the vehicle-treated group compared with that in the control group. There were significant differences between the vehicle-, Pio-, and CD-treated groups. Values are means ± SE.

**Fig. 2. Plasminogen activator inhibitor-1 (PAI-1) protein levels.** A: representative Western blot analysis of PAI-1. Top: PAI-1 protein bands. Bottom: β-actin protein bands. B: PAI-1/β-actin protein ratio according to band density. The PAI-1/β-actin protein ratio was significantly increased in the vehicle-treated group and significantly decreased in the pioglitazone (Pio)- and candesartan (CD)-treated groups compared with that in the control group. Values are means ± SE.
group (Pio-treated group: 4.8 ± 1.3 μU/ml, \( P = 0.07 \); CD-treated group: 4.2 ± 0.5 μU/ml, \( P < 0.05 \) vs. the vehicle-treated group). Similar results were found when the relative plasmin activity was assessed in casein gel zymographic bands (control group: 1.0 ± 0.1 vs. vehicle-treated group: 9.5 ± 2.1, \( P < 0.01 \); Pio-treated group: 1.9 ± 0.3, \( P < 0.01 \); CD-treated group: 2.2 ± 0.4, \( P < 0.01 \) vs. the vehicle-treated group) (Fig. 4). The intrarenal distribution of plasmin activity according to in situ zymography is shown in Fig. 5. Plasmin activity increased with age in the tubules and the interstitial area (Fig. 5, A and B), and this increase was inhibited by treatment with Pio or CD (Fig. 5, C and D).

**Immunostaining of macrophages.** Interstitial ED1-positive macrophages were rare in the control group. Prominent interstitial infiltration of macrophages was observed in the vehicle-treated group, and this infiltration was attenuated in the Pio- and CD-treated groups (Fig. 6A). Quantification for the number of the ED1-positive interstitial macrophages is shown in Fig. 6B. The number of interstitial macrophages was significantly increased in the vehicle-treated group compared with those in the control group (75.0 ± 7.8 vs. 14.9 ± 1.7 cells, \( P < 0.01 \)). Among the 32-wk-old rats, the number of the ED1-positive interstitial macrophages was significantly reduced in both the Pio- and CD-treated groups compared with those in the vehicle-treated group (Pio-treated group: 47.5 ± 2.5 cells, \( P < 0.01 \); CD-treated group: 44.2 ± 2.9 cells, \( P < 0.01 \) vs. vehicle-treated group).

**DISCUSSION**

SHC rats begin to show urinary protein from ~8 wk of age and progress to renal failure by ~30 wk of age. Thus the SHC rat model reflects chronic progressive kidney disease. Results of the present study indicate that the long-term administration of Pio or CD protects against chronic progressive renal failure. Administration of Pio and CD was initiated at 8 wk of age and was continued for 24 wk. Blood pressure increased with age. However, this increase was inhibited by treatment with Pio or CD. At 16 wk of age, there was no difference in blood pressure between the vehicle- and Pio-treated groups, whereas there was a significant decrease in the CD-treated group. Blood pressure increased sharply after 16 wk of age in the vehicle-treated group, but this increase was not observed in the Pio- or CD-treated groups. Thus Pio appears to exert primarily a renoprotective effect independently of blood pressure; the antihypertensive effect of Pio was not evident in the early phase of treatment. CD appears to exert both renoprotective and antihypertensive effects; the causal relation between these effects is not known.

The renoprotective effect of ARBs against clinical or experimental renal disease has been widely reported (7, 22, 26). Long (29)-and short-term (21) renoprotective effects of ARBs have also been reported in SHC rats. Rodriguez-Iturbe et al. (29) reported that treatment with an ARB (olmesartan) for 6 mo prevented proteinuria, renal failure, hyperlipidemia, and glomerulosclerosis in SHC rats, consistent with our results. Matsuo et al. (21) reported that treatment with CD for 6 wk inhibited proteinuria, hypercholesterolemia, and histological changes in SHC rats. Many mechanisms regarding the renoprotective effects of ARB have been suggested, including a decrease in blood pressure, reduction in urinary protein, and...
inhibition of PAI-1 expression. These effects were also found in response to CD treatment in the present study.

On the contrary, little information is available regarding the long-term effects of PPARγ agonists in nondiabetic renal disease. A single report indicated a renoprotective effect of Pio in rats with progressive nephropathy (1), consistent with our results. In that report, a significant reduction in proteinuria and a renoprotective effect of Pio similar to that induced by CD was observed in passive Heymann nephritis. Transcriptional upregulation of the nephrin gene by Pio was proposed as the mechanism of the antiproteinuric effect. There is also a report that a PPARγ agonist protects podocytes against damage in progressive glomerulosclerosis with urinary protein (13). Recent evidence indicates that a decreased urinary protein level attenuates tubulointerstitial damage and protects from the progression of renal disease (10, 11, 33). The urinary protein level in SHC rats was also prominently decreased by Pio in the present study.

Progressive renal disease is frequently characterized by overexpression of PAI-1, suggesting its role in renal fibrosis (25, 27). It has also been reported that the renin-angiotensin-aldosterone system causes renal fibrosis via PAI-1, and the inhibition of this system by ARB inhibits PAI-1 expression and progression of renal fibrosis (2). Ma et al. (18) reported that treatment with a PPARγ agonist was associated with decreased PAI-1 expression and exerted a renoprotective effect on nondiabetic glomerulosclerosis in rats. Our present results also showed that the PAI-1 protein level was significantly increased in the vehicle-treated group compared with that in the control group, and the PAI-1 protein level was significantly decreased in both the Pio- and CD-treated groups compared with that in the vehicle-treated group. Thus, in the present study, a PPARγ agonist and an ARB improved renal fibrosis, at least in part, via inhibition of PAI-1. Two mechanisms have been suggested regarding how PAI-1 exerts a profibrotic effect in progressive kidney disease: regulation of protease (mainly plasmin) activity and facilitation of macrophage migration (4). Although the PAI-1 level in renal disease has been analyzed extensively, few reports deal with plasmin activity level in renal disease. There are no reports regarding local plasmin activity in renal tissue in situ (24). Plasmin is expected to decrease with the upregulation of PAI-1. In the present study, PAI-1 and plasmin activity increased concomitantly with the progression of renal fibrosis, indicating that the profibrotic action of PAI-1 is not due to inhibition of plasmin activity. In this respect, Edgerton et al. (5) reported that plasmin(ogen) deficiency improved renal interstitial fibrosis in mice with unilateral ureteral obstruction (UUO), suggesting a function of PAI-1 independent of plasmin generation. Matsuo et al. (20) also suggested that PAI-1 is directly involved in interstitial fibrosis and tubular damage in the UUO model in PAI-1-overexpressing mice. The concomitant increase in plasmin and PAI-1 in SHC rats with renal fibrosis in the present study may be attributable to the alteration of proteins other than PAI-1 that regulate plasmin activity, such as α2-antiplasmin. A secondary action of PAI-1 against excess plasmin activity in aged SHC rats may also be involved.

Decreased PAI-1 appears to improve renal fibrosis not through protease regulation. We observed that macrophage infiltration (ED1 staining) was significantly decreased in both the Pio- and CD-treated groups compared with that in the vehicle-treated group. We suspect that these drugs inhibit...
PAI-1 expression, thereby attenuating the cellular infiltration of macrophages and leading to a reduction of renal fibrosis.

In conclusion, we identified a significant increase in renal PAI-1 expression in association with the progression of renal fibrosis in SHC rats. Long-term treatment with Pio or CD decreased proteinuria, renal PAI-1 expression, and inhibited the progression of renal fibrosis. Plasmin activity was also decreased by Pio or CD. Thus the profibrotic effect of PAI-1 in this model appears to be independent of its regulatory function in the plasmin cascade. It was indicated that the renoprotective effects by Pio and CD were attributable to the pathways, at least in part, via decreased urinary protein level and inhibition of PAI-1 expression, leading to the cellular infiltration of macrophages.

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