Original Article

Pioglitazone Enhances the Antihypertensive and Renoprotective Effects of Candesartan in Zucker Obese Rats Fed a High-Protein Diet

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The metabolic syndrome is a risk factor for the development of chronic kidney disease. Angiotensin II type 1 receptor blockers (ARBs) and thiazolidinediones (TZDs) provide renovascular protection, probably in the metabolic syndrome. However, the effect of both agents administered together in patients with metabolic syndrome remains to be determined. The aim of this study was to assess the effects of ARB plus TZD combination therapy in Zucker obese rats fed a high-protein diet, an animal model of metabolic syndrome and renal injury. Zucker obese rats were fed a high-protein diet (OHP; $n=6$), a high-protein diet containing candesartan, an ARB (OHP+C; $n=6$), or a high-protein diet containing both candesartan and pioglitazone (OHP+CP; $n=6$) for 12 weeks. Systolic blood pressure and urinary protein excretion were measured throughout the study, and renal histology and immunohistochemistry were assessed at 12 weeks. OHP rats developed hypertension (157 ± 4 mmHg) and proteinuria (178 ± 44 mg/d), and these conditions were significantly ameliorated by candesartan (to 143 ± 3 mmHg and 84 ± 25 mg/d, respectively). Pioglitazone enhanced the antihypertensive and anti-proteinuric effects of candesartan (121 ± 3 mmHg, 16 ± 8 mg/d, respectively). Histologically, candesartan ameliorated glomerulosclerosis, podocyte injury, interstitial fibrosis and monocyte/macrophage infiltration into the tubulointerstitium in the kidneys of OHP rats. Pioglitazone abrogated residual interstitial fibrosis in the kidneys of OHP+C rats. Our results suggested that pioglitazone augmented the antihypertensive, anti-proteinuric and possibly renal anti-fibrotic actions of candesartan in Zucker obese rats fed a high-protein diet. The combination therapy of ARB and TZD may protect against renal injury in patients with metabolic syndrome. (*Hypertens Res* 2008; 31: 745–755)

Key Words: dietary protein, angiotensin II type 1 receptor blocker, thiazolidinediones, tubulointerstitial injury

Introduction

Previous studies from the United States and Japan have indicated that patients with metabolic syndrome are at increased risk of morbidity and mortality associated with cardiovascular disease (CVD) and the development of chronic kidney disease (CKD) (1–5). Several experimental and clinical studies have documented the renoprotective effects of inhibitors of

the renin-angiotensin-aldosterone system (RAAS), including angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II type 1 receptor blockers (ARBs), and their potential usefulness for retarding the development of CKD in patients with the metabolic syndrome (6, 7). In addition, recent studies have revealed that thiazolidinediones (TZDs), which are activators of the transcriptional factor peroxisome proliferator-activated receptor- γ (PPAR- γ), have antihypertensive and renoprotective effects as well as the ability to improve insulin

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resistance and dyslipidemia (8, 9). At this stage, however, the incidence of CKD has not diminished despite the general use of these agents (10). Therefore, it is important to consider a strategy to reduce CKD that is associated with the metabolic syndrome, because CKD is also a risk factor for the development of CVD and, consequently, for the increased morbidity and mortality associated with this disease (11).

Zucker obese rats exhibit hyperlipidemia, peripheral insulin resistance, glucose intolerance, and mild to severe hyperglycemia, and are therefore considered a suitable animal model of the metabolic syndrome (12, 13). They also spontaneously develop proteinuria and focal glomerulosclerosis (FGS) with advancing age (14). In this model, a high-protein diet exacerbates proteinuria and renal histological injury at an earlier age compared with a standard-protein diet (15). Therefore, Zucker obese rats fed a high-protein diet can be considered useful to elucidate the pathogenetic mechanisms involved in the development of CKD in association with the metabolic syndrome.

There are evidences to suggest that ARBs and ACEIs prevent the development of renal injury in diabetic and obese Zucker rats (16, 17). TZDs also have been shown to ameliorate renovascular injuries in Wistar or Zucker obese rats (18, 19). A previous study reported that rosiglitazone, a TZD compound, enhanced the renoprotective effects of cilazapril, an ACEI, in Zucker diabetic rats (20). To our knowledge, however, there is no information on whether TZD augments the renoprotective effects of ARB in an animal model of the metabolic syndrome.

In the present study, we investigated whether pioglitazone, a TZD agent, would augment the antihypertensive and renoprotective effects of candesartan, an ARB, in Zucker obese rats fed a high-protein diet, and compared the effects of candesartan and pioglitazone combination therapy with candesartan monotherapy in the same model. The purpose of this study was to validate the effects of TZD and ARB combination therapy against the development of CKD in association with the metabolic syndrome.

Methods

Animal Preparation

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Kawasaki Medical School, Kurashiki, Japan (No. 05-018). Male Zucker lean and obese rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). They were housed in a temperature- and humidity-controlled room with 12/12 h light/dark cycles and had free access to water. Twelve-week-old obese rats weighing ~550 g were randomly divided into four groups ($n=6$ in each group) comprising obese rats fed on high-protein chow (OHP), high-protein chow with tap water containing hydralazine (5 mg/kg/d; Sigma-Aldrich Japan, Tokyo, Japan) (OHP+H), high-protein

Table 1. Content of Standard-Protein and High-Protein Chows

Ingredients	Standard protein	High protein
Protein, g/100 g	23.6	42.4
Fat, g/100 g	5.3	7.2
Ash, g/100 g	6.1	3.6
Fiber, g/100 g	2.9	8.0
Carbohydrates, g/100 g	54.4	27.2
Gross energy, kcal/100 g	360	343

chow containing candesartan (1 mg/kg/d; Takeda, Osaka, Japan) (OHP+C) or high-protein chow containing both candesartan and pioglitazone (2.5 mg/kg/d; Takeda) (OHP+CP). Table 1 lists the ingredients of the standard-protein chow (MF; Oriental Yeast, Osaka, Japan) and high-protein chow, prepared by modifying AIN-93G (Oriental Yeast). Age-matched lean rats weighing ~350 g were fed standard-protein chow (LSP; $n=6$). Body weight and food intake per day were measured every week. Twelve weeks after the start of the dietary regimen, rats were killed by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The kidneys were removed from all animals for histological analyses, fixed in 4% paraformaldehyde and embedded in paraffin.

Physiological and Biochemical Measurements

Systolic arterial blood pressures (BP) were measured in prewarmed rats by the tail-cuff method (BP-98A; Softron, Tokyo, Japan) (21). To collect urine samples at 6 and 12 weeks, rats were placed in metabolic cages for 24 h and provided tap water but no food. The urinary protein level was measured using the pyrogallol sulfonphthalein method. Rats were sacrificed after urine collection, and blood samples were obtained immediately. Serum creatinine, total cholesterol, fasting glucose and fasting insulin levels were measured.

Histological Assessment

Four- μ m-thick sections were prepared from renal tissue samples embedded in paraffin, and stained with periodic acid-Schiff (PAS) and Masson-trichrome (MT). Kidney sections were photographed using a Nikon Eclipse E800 Microscope (Nikon, Tokyo, Japan) and digitalized into 1372–1024 pixel color scale images using the Nikon ACT-1C DXM 1200C program (Nikon). Histological scores were assessed using a Nikon Coolscope (Nikon). Two pathologists performed semi-quantitative analysis of PAS-stained and immunostained sections in a blind fashion. The net score for each rat was taken as the average of the scores obtained for that rat by the two pathologists. The severity of glomerular injury was evaluated by the glomerulosclerosis (GS) score as described previously (22): 0, no sclerosis; 1, sclerotic change in less than 25% of the total area of the glomerulus; 2, from 25% to 50% of the

Fig. 1. Temporal profiles of body weight (A) and food intake per day (B). At 12 weeks, body weight was significantly increased in the obese rat groups compared with LSP rats ($p < 0.05$). It was not different between OHP and OHP+C rats, but was significantly lower in OHP+H rats than in OHP rats ($p < 0.05$). Body weight was higher in OHP+CP rats than in OHP and OHP+C rats, albeit insignificantly. Food intake was significantly higher in the obese rat groups than in LSP rats ($p < 0.05$). It was not different among OHP, OHP+H and OHP+CP rats, while it was significantly higher in OHP+C rats than in OHP rats ($p < 0.05$). Data are the means of the results for 6 rats in each group.

total area; 3, from 50% to 75% of the total area; 4, in more than 75% of the total area. At least 50 glomeruli were randomly selected in each rat and the mean score was calculated. The severity of tubulointerstitial injury was evaluated by the interstitial fibrosis (IF) score, as described previously (22). The fibrotic areas stained blue by MT were measured quantitatively by a pathologist using a color image analyzer (Win ROOF; Mitani, Fukui, Japan). The percentage of interstitial fibrotic areas per cortical field (magnification, $\times 100$) was calculated, and the mean percentage in 10 randomly selected fields was determined as the IF score for each rat.

Immunohistochemical Study

Immunohistochemical analysis was performed for detection of desmin, α -smooth muscle actin (α -SMA) and ED1 by the method described previously (22). A mouse anti-desmin monoclonal antibody (Dako, Glostrup, Denmark), a mouse anti- α -SMA monoclonal antibody (Sigma, St. Louis, USA), a mouse anti-ED1 monoclonal antibody (Serotec, Oxford, UK) and a rabbit anti-fibronectin polyclonal antibody (Chemicon, Temecula, USA) were used as the primary antibodies. Antibody binding was detected using a Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine (Sigma-Aldrich Japan). In the case of immunostaining for desmin, the outer cell layer of the glomerular tuft was evaluated to determine the desmin score, as described previously (23): 0, from 0% to 5% staining; 1, from 5% to 25%; 2, from 25% to 50%; 3, from 50% to 75%; 4, greater than 75%. At least 50 glomeruli were randomly selected in each rat and the mean score was calculated. In immunostaining for α -SMA and fibronectin, the positive area in a tubulointerstitial field (magnification, $\times 100$) was graded semiquantitatively by following the previously reported method (23): 0, from 0% to 5% staining; 1, from 5% to 25%;

2, from 25% to 50%; 3, from 50% to 75%; 4, greater than 75%. The mean score in 10 randomly selected fields was determined as the α -SMA and fibronectin scores for each rat. In the case of immunostaining for ED1, the number of positive cells per field (magnification, $\times 100$) in the tubulointerstitium was evaluated and the mean number in 10 randomly selected fields was determined for each rat.

Statistical Analysis

Values are expressed as the means $\pm$ SEM. All parameters were evaluated with the two-tailed unpaired Welch's t -test or Mann-Whitney's U test, or compared by one-way analysis of variance (ANOVA) when multiple mean comparisons were required. A p value less than 0.05 was considered statistically significant.

Results

Physiological and Biochemical Parameters

The temporal profiles of body weight and daily food intake are shown in Fig. 1. At 12 weeks after the initiation of various diet regimens, body weight (Fig. 1A) was significantly higher in the obese rat groups compared with the LSP rats (LSP 484 ± 14 g, $p < 0.05$). The body weight was not different between the OHP (874 ± 14 g) and OHP+C rats (874 ± 23 g), while it was significantly lower in OHP+H rats compared with OHP rats (805 ± 18 g, $p < 0.05$). Body weight tended to be higher in OHP+CP rats than in OHP and OHP+C rats, but the difference was not significant (986 ± 60 g). Food intake per day (Fig. 1B) was significantly higher in the obese rat groups than in LSP rats (LSP 21 ± 1 g/d, $p < 0.05$). It was not significantly different among OHP, OHP+H and OHP+CP rats (28 ± 1 , 27 ± 2 , 29 ± 3 g/d, respectively), while it was sig-

Table 2. Systolic Blood Pressure and Urinary Protein Excretion at 6 and 12 Weeks after Intake of Special Diets

	LSP	OHP	OHP+H	OHP+C	OHP+CP
6 weeks					
Systolic blood pressure, mmHg	150±5	142±3	124±6 [†]	126±5 [†]	120±3 [‡]
Urinary protein, mg/d	11±1	51±19	53±21	39±23	9±3
12 weeks					
Systolic blood pressure, mmHg	137±6	157±4*	134±7 [†]	143±3 [†]	121±3 [‡]
Urinary protein, mg/d	39±12	178±44*	210±39	84±25	16±8 [‡]

LSP, Zucker lean rats fed standard-protein chow; OHP, Zucker obese rats fed high-protein chow; OHP+H, OHP rats on hydralazine-containing tap water; OHP+C, Zucker obese rats fed high-protein chow containing candesartan; OHP+CP, Zucker obese rats fed high-protein chow containing both candesartan and pioglitazone. Data are mean±SEM. $n=6$ in each group. * $p<0.05$ vs. LSP, [†] $p<0.05$ vs. OHP, [‡] $p<0.05$ vs. OHP and OHP+C.

Table 3. Biochemical Characteristics at 12 Weeks after Intake of Special Diets

	LSP	OHP	OHP+H	OHP+C	OHP+CP
Creatinine, mg/dL	0.25±0.01	0.33±0.06	0.19±0.01	0.27±0.05	0.18±0.01
Creatinine clearance, mL/min	5.3±0.2	3.0±0.5*	4.8±0.3 [†]	4.0±0.7	5.0±0.4 [†]
Total cholesterol, mg/dL	88±6	244±25*	282±43	185±18 [†]	122±9 [‡]
Fasting glucose, mg/dL	151±7	491±91*	194±14 [†]	318±61	211±33 [†]
Fasting insulin, ng/mL	0.4±0.1	4.4±1.2*	4.2±0.6	5.3±0.9	5.7±1.3

LSP, Zucker lean rats fed standard-protein chow; OHP, Zucker obese rats fed high-protein chow; OHP+H, OHP rats on hydralazine-containing tap water; OHP+C, Zucker obese rats fed high-protein chow containing candesartan; OHP+CP, Zucker obese rats fed high-protein chow containing both candesartan and pioglitazone. Data are mean±SEM. $n=6$ in each group. * $p<0.05$ vs. LSP, [†] $p<0.05$ vs. OHP, [‡] $p<0.05$ vs. OHP and OHP+C.

nificantly higher in OHP+C rats than in OHP rats (31 ± 1 g/d, $p<0.05$). Left kidney weight was significantly increased in OHP, OHP+H, OHP+C and OHP+CP rats (2.30 ± 0.11 , 2.14 ± 0.14 , 2.15 ± 0.12 , 1.97 ± 0.09 g, respectively) compared with LSP rats (1.52 ± 0.07 g, $p<0.05$) and it was significantly different among the obese rat groups.

Table 2 summarizes changes in systolic blood pressure (SBP) and urinary protein excretion (UPE) at 6 and 12 weeks after the initiation of various diet regimens. At 6 weeks, SBP was significantly decreased in OHP+H, OHP+C and OHP+CP rats compared with OHP rats ($p<0.05$, each). OHP+CP rats had a lower mean SBP than OHP+H and OHP+C rats, but the differences were not significant. UPE was higher in OHP rats than in LSP rats, but not significantly. It was lower in OHP+C than in OHP rats. The level of UPE in OHP+CP rats was approximately equal to that in LSP rats. At 12 weeks, SBP was significantly higher in OHP rats than in LSP rats ($p<0.05$). The elevation of SBP in OHP rats was significantly prevented in OHP+H, OHP+C and OHP+CP rats ($p<0.05$, each). The SBP in OHP+CP rats was significantly lower than that in OHP+C rats ($p<0.05$). UPE was significantly increased in OHP rats compared with LSP rats ($p<0.05$ vs. LSP). It was lower in OHP+C rats, but not in OHP+H rats, than in OHP rats. In OHP+CP rats, UPE was

significantly lower than in OHP rats ($p<0.05$). UPE in OHP+CP rats was significantly lower than that in OHP+C rats ($p<0.05$). These data suggest that pioglitazone enhanced the antihypertensive and the urinary protein-lowering effects of candesartan.

Table 3 summarizes the biochemical data at 12 weeks after the initiation of various diet regimens. The creatinine level was not different among the groups. Creatinine clearance was significantly lower in OHP rats compared with LSP rats ($p<0.05$). It was significantly increased in OHP+H and OHP+CP rats compared with OHP rats ($p<0.05$), but insignificantly in OHP+C rats. The total cholesterol level was significantly higher in OHP rats compared with LSP rats ($p<0.05$). It was significantly lower in OHP+C and OHP+CP rats, but not in OHP+H rats, compared with OHP rats ($p<0.05$). The total cholesterol level in OHP+CP rats was significantly lower than that in OHP+C rats ($p<0.05$). The fasting glucose level was significantly higher in OHP rats compared with LSP rats ($p<0.05$). It was significantly decreased in OHP+H and OHP+CP rats, but not in OHP+C rats, compared with OHP rats ($p<0.05$). The fasting insulin level was significantly higher in the obese rat groups compared with the LSP rats ($p<0.05$ vs. LSP). It was not significantly different among the obese rat groups.

Fig. 2. Histological findings of glomeruli in tissue sections stained by periodic acid-Schiff (A–E) and glomerulosclerosis (GS) score (F). The kidneys of LSP rats showed minor glomerular abnormalities (A). The kidneys of OHP rats exhibited segmental sclerosis in glomeruli (arrowheads), accompanied with proliferation of smooth muscle cells in the afferent arterioles (arrows) (B). The degree of glomerular sclerosis was lower in the OHP+H-group kidneys (C) compared with the OHP-group kidneys. In the OHP+C-group kidneys, slight segmental sclerosis was observed in the glomeruli (D), while the OHP+CP-group kidneys showed minor glomerular abnormalities (E). Bar = 100 μ m. $n = 6$ rats in each group. * $p < 0.05$ vs. LSP, † $p < 0.05$ vs. OHP.

Histological and Immunohistochemical Studies in Glomeruli

Representative histological findings of glomeruli in all groups are depicted in Fig. 2A–E. The kidneys of LSP rats showed minor glomerular abnormalities (Fig. 2A). The kidneys of OHP rats exhibited segmental sclerosis in the glomeruli, accompanied with proliferation of smooth muscle cells in the afferent arterioles (Fig. 2B). The degree of glomerular sclerosis was lower in the OHP+H-group kidneys compared with the OHP-group kidneys (Fig. 2C). In the kidneys of the OHP+C group, slight segmental sclerosis was observed in the glomeruli (Fig. 2D), while the kidneys of the OHP+CP group showed minor glomerular abnormalities (Fig. 2E). The GS score was significantly higher in OHP rats (0.82 ± 0.05) compared with LSP rats (0.37 ± 0.02 , $p < 0.05$; Fig. 2F). It was significantly lower in OHP+H (0.58 ± 0.03), OHP+C (0.52 ± 0.05) and OHP+CP rats (0.47 ± 0.03) compared with OHP rats ($p < 0.05$, each), but not significantly different between OHP+C and OHP+CP rats.

The immunohistochemical findings for desmin in all groups are depicted in Fig. 3A–E. Increased expression of desmin in podocytes reflects podocyte injury (23). The LSP-group kidneys showed positive staining only in arterioles (Fig. 3A). Positive staining was detected in podocytes as well as arterioles in the kidneys of the OHP (Fig. 3B) and OHP+H

groups (Fig. 3C). Both the amount and intensity of positive staining in podocytes were distinctly increased in the kidneys of OHP and OHP+H rats compared with those of LSP rats. In OHP+C-group kidneys, positive staining in the glomerular tuft was lower than in OHP-group kidneys (Fig. 3D). Notably, positive staining at the glomerular edge was weak in the OHP+C-group kidneys. In the kidneys of the OHP+CP rats, positive staining had almost disappeared in the glomerular tuft, although it remained only in the afferent arterioles (Fig. 3E). The desmin score was significantly higher in OHP rats (1.39 ± 0.03) than in LSP rats (0.53 ± 0.09 , $p < 0.05$, Fig. 3F), and significantly lower in OHP+C (0.48 ± 0.10) and OHP+CP rats (0.26 ± 0.03), but not in OHP+H rats (1.44 ± 0.12), compared with OHP rats ($p < 0.05$, each). The score in OHP+CP rats was lower than that in OHP+C rats, albeit insignificantly. These results suggest that candesartan, when administered alone, improved both glomerulosclerosis and podocyte injury in OHP rats to levels close to those seen in LSP rats.

Histological and Immunohistochemical Studies in the Tubulointerstitium

Figure 4A–E provides representative histological findings of the tubulointerstitium in all groups. The kidneys of OHP rats exhibited mild to moderate tubulointerstitial damage with

Fig. 3. Immunohistochemical findings for desmin in the glomeruli (A–E) and desmin score (F). The kidneys of LSP rats showed positive staining only in the arterioles (arrow) (A). Positive staining was detected in podocytes (arrowheads) as well as arterioles in OHP-group (B) and OHP+H-group kidneys (C). In the kidneys of OHP+C rats, positive staining in the glomerular tuft was lower than in the kidneys of OHP rats (D). Notably, positive staining at the glomerular edge was weak in the OHP+C group. In the OHP+CP-group kidneys, positive staining almost disappeared in the glomerular tuft, although it remained only in the afferent arterioles (E). Bar = 100 μ m. $n = 6$ rats in each group. * $p < 0.05$ vs. LSP, † $p < 0.05$ vs. OHP.

tubular atrophy and interstitial fibrosis (Fig. 4B) whereas those of the LPS rats did not (Fig. 4A). The kidneys of OHP+H rats showed tubulointerstitial damage similar to that in the kidneys of OHP rats (Fig. 4C). In the kidneys of OHP+C rats, interstitial fibrosis was still seen in the sections of the pericapsular area and tubulointerstitium, accompanied with slight tubular changes (Fig. 4D). The kidneys of OHP+CP rats showed minor abnormalities in the tubulointerstitium (Fig. 4E). The IF score was significantly higher in OHP rats ($13.9 \pm 1.2\%$) than in LSP rats ($5.9 \pm 0.5\%$, $p < 0.05$, Fig. 3F). It was significantly lower in OHP+C ($6.5 \pm 0.2\%$) and OHP+CP rats ($4.6 \pm 0.3\%$), but not in OHP+H rats ($11.7 \pm 1.0\%$), compared with OHP rats ($p < 0.05$, each). Of importance, the IF score in OHP+CP rats was significantly lower than that in OHP+C rats.

The immunohistochemical findings for α -SMA in all groups are depicted in Fig. 5A–E. In the diseased kidney, *de novo* expression of α -SMA represents activation of mesangial cells and interstitial (myo-) fibroblasts (23). The LSP-group kidneys showed positive staining only in arterioles (Fig. 5A). In the OHP-group kidneys, positive staining was detected in the interstitial area as well as the arterioles (Fig. 5B). The extent of positive staining in the interstitial area was distinctly increased in the OHP-group kidneys compared with the LPS-group kidneys. Positive staining in the glomeruli was also slightly increased in the kidneys of the OHP group. In the kid-

neys of OHP+H rats, positive staining remained in the interstitial area (Fig. 5C). In the kidneys of the OHP+C group, positive staining was restricted to parts of the pericapsular and peritubular interstitial areas (Fig. 5D). The kidneys of OHP+CP rats showed little positive staining, with the exception of the arterioles (Fig. 5E). The α -SMA score was higher in the OHP (0.66 ± 0.28) and OHP+H rats (0.53 ± 0.13) compared with the LSP rats (0.05 ± 0.03), but not significantly (Fig. 5F). The OHP+C rats showed a lower score compared with the OHP rats (0.20 ± 0.11). The score in OHP+CP rats was also similar to that in LSP rats (0.05 ± 0.05).

The immunohistochemical findings for ED1 in all groups are depicted in Fig. 6A–E. ED1-positive cells reflect monocytes/macrophages, which infiltrate into renal tissues at the site of inflammation. The number of ED1-positive cells was significantly higher in OHP rats (4.6 ± 1.2) than in LSP rats (0.7 ± 0.1 , $p < 0.05$, Fig. 3F). It was significantly decreased in OHP+C (1.1 ± 0.2) and OHP+CP rats (1.1 ± 0.3), but not in OHP+H rats (3.7 ± 0.6), compared with OHP rats ($p < 0.05$, each). The number of ED1-positive cells was not different between OHP+C and OHP+CP rats. The number of ED1-positive cells in glomeruli was not significantly different between LSP and OHP rats (data not shown).

To confirm the therapeutic effects of the combination therapy on interstitial fibrosis, the immunohistochemical evaluation of fibronectin, one of the extracellular matrix (ECM)

Fig. 4. Histological findings of the tubulointerstitium in tissue sections stained by Masson-trichrome (A–E) and interstitial fibrosis (IF) score (F). The kidneys of the OHP rats exhibited mild to moderate tubulointerstitial damage with tubular atrophy and interstitial fibrosis (arrowheads) (B), while those of the LSP group showed none of these changes (A). The kidneys of the OHP+H rats showed tubulointerstitial damage similar to that of the OHP-group kidneys (C). In the OHP+C-group kidneys, interstitial fibrosis was still seen in sections of the pericapsular area and tubulointerstitium, accompanied with slight tubular changes (D). The OHP+CP-group kidneys showed minor abnormalities in the tubulointerstitium (E). Bar = 100 μ m. $n = 6$ rats in each group. * $p < 0.05$ vs. LSP, † $p < 0.05$ vs. OHP, § $p < 0.05$ vs. OHP and OHP+C.

proteins, was performed. The kidneys of the LSP rats showed little positive staining in the interstitium (Fig. 7A). In the OHP-group kidneys, the positive staining was widely expanded in the interstitium (Fig. 7B). Positive staining was observed in parts of the peritubular and pericapsular interstitial areas in the kidneys of OHP+H rats (Fig. 7C) and was slightly apparent in the interstitial areas in the kidneys of OHP+C rats (Fig. 7D). In OHP+CP-group kidneys, the positive staining in the interstitium was abrogated completely (Fig. 7E). The fibronectin score was significantly higher in the kidneys of OHP rats (0.78 ± 0.14) than in those of LSP rats (0.25 ± 0.05 , $p < 0.05$, Fig. 7F). It was significantly decreased in OHP+C-group kidneys (0.30 ± 0.06) and OHP+CP-group kidneys (0.10 ± 0.07) compared with OHP-group kidneys ($p < 0.05$, each), but not significantly changed in OHP+H-group kidneys (0.42 ± 0.07). Notably, the score was decreased in the kidneys of OHP+CP rats compared with those of OHP+C rats, albeit insignificantly. These findings suggest that pioglitazone abrogated residual interstitial fibrosis in the candesartan-treated kidneys, although candesartan monotherapy sufficiently inhibited monocyte/macrophage infiltration, ECM production and possibly (myo-)fibroblast activation in the tubulointerstitium.

Discussion

In the present study, candesartan prevented the development of proteinuria, glomerulosclerosis, podocyte injury, interstitial fibrosis and monocyte/macrophage infiltration into the tubulointerstitium as well as elevation of BP in Zucker obese rats fed a high-protein diet, while hydralazine ameliorated only glomerulosclerosis *via* an antihypertensive effect. Thus, pioglitazone enhanced the antihypertensive action and urinary protein-lowering effects of candesartan in this model. Moreover, pioglitazone may have augmented the anti-fibrotic effect of candesartan in the kidney. These results suggest that combination therapy with an ARB and a TZD could be more effective at conferring renovascular protection than ARB monotherapy in patients with metabolic syndrome.

At 12 weeks after the initiation of various diet regimens, combination therapy with candesartan and pioglitazone significantly reduced proteinuria in OHP rats, while candesartan monotherapy failed to produce such an effect (Table 2). In addition, the antihypertensive effect of the combination therapy was significantly greater than that of the monotherapy. ARBs are known to reduce proteinuria not only by decreasing systemic BP but also by lowering intraglomerular pressure through the dilation of efferent arterioles (24). TZDs were

Fig. 5. Immunohistochemical findings for α -smooth muscle actin (α -SMA) in the tubulointerstitium (A–E) and α -SMA score (F). The kidneys of the LSP group showed positive staining only in the arterioles (A). In the kidneys of the OHP group, positive staining was detected in the interstitial area (arrows) as well as the arterioles (B). Positive staining in the glomeruli was also slightly increased in the OHP-group kidneys. In the OHP+H-group kidneys, positive staining was apparent in the interstitial area (C). In the OHP+C-group kidneys, positive staining was restricted to parts of the pericapsular and peritubular interstitial areas (D). The kidneys of the OHP+CP rats showed little positive staining, except in the arterioles (E). The α -SMA score was not significantly different among all groups. Bar = 100 μ m. a, arterioles; g, glomerulus. n = 6 rats in each group.

reported to attenuate the development of hypertension in Dahl salt-sensitive rats and to inhibit proliferation of renal arteriolar smooth muscle cells (25). Furthermore, TZDs also ameliorated endothelial dysfunction of preglomerular arterioles in Zucker obese rats and diet-induced type 2 diabetic mice (26, 27). Improved endothelial function in intrarenal arteries and arterioles, accompanied by a fall in systemic BP, may account for the enhanced reduction in proteinuria by the ARB plus TZD combination therapy. The improvement of endothelial function by TZDs is reported to be at least partially independent of increased insulin sensitivity (28). It is associated with reduced oxidative stress (29, 30), increased endothelial nitric oxide (NO) production (31, 32) or decreased systemic asymmetric dimethylarginine, which is followed by restored endothelial NO production (33). Intriguingly, one *ex vivo* study revealed that troglitazone, a TZD compound, dilated not only the afferent but also the efferent arterioles (34). In this regard, it is noteworthy that upregulation of the angiotensin II type 1 receptor (AT1R) was reported in the kidneys of Zucker obese rats (35). In addition, a TZD was reported to prevent the upregulation of AT1R induced by angiotensin II in the vasculature (36). TZDs may thus enhance the ARB-induced dilation of the efferent arterioles by decreasing the AT1R expression in the efferent arterioles.

Podocyte injury plays a critical role in the development of

proteinuria in diabetic nephropathy (37). In the kidneys of Zucker obese rats, podocyte injury was detected at an early age with increased desmin staining in the glomerular tuft and edge (23). In our study, Zucker obese rats with a high-protein diet showed increased desmin staining in the glomerular tuft and edge. On the other hand, candesartan reduced the desmin staining and markedly decreased the desmin score. The combination of candesartan and pioglitazone cancelled the desmin staining, although the desmin score was not significantly different between the combination therapy and monotherapy. The latter finding was probably because candesartan monotherapy was sufficiently effective to improve podocyte injury in this model, at least when assessed by desmin staining. One of the causative mechanisms of proteinuria is down-regulation of nephrin, which exists in the slit diaphragm of podocytes (37). The enhanced reduction of proteinuria by the combination therapy in the present study may have been associated with an overexpression of nephrin, since pioglitazone has been reported to exert an anti-proteinuric effect through the enhancement of nephrin gene transcription (38).

The IF scoring suggested that pioglitazone augmented the anti-fibrotic action of candesartan in the kidneys of OHP rats, although the α -SMA and fibronectin scores were not significantly different between the group receiving candesartan plus pioglitazone combination therapy and that receiving cande-

Fig. 6. Immunohistochemical findings for ED1 in the tubulointerstitium (A–E) and the number of ED1-positive cells per field (F). The positive cells (arrows) were increased in kidneys of the OHP group (B) compared with those of the LSP group (A). They were fewer in number in the OHP+H-group kidneys (C) than in the OHP-group kidneys. Few positive cells were observed in the kidneys of the OHP+C (D) and OHP+CP (E) groups. Bar = 100 μ m. g, glomerulus. $n = 6$ rats in each group. * $p < 0.05$ vs. LSP, † $p < 0.05$ vs. OHP.

Fig. 7. Immunohistochemical findings for fibronectin in the interstitium (A–E) and fibronectin score (F). The kidneys of the LSP group showed little positive staining in the interstitium (A). In the OHP-group kidneys, the positive staining was expanded widely in the interstitium (B). It was observed in parts of the peritubular and pericapsular interstitial areas (arrows and arrowheads, respectively) in the OHP+H-group kidneys (C) and was slightly present in the interstitial area (arrows) in OHP+C-group kidneys (D). In kidneys of the OHP+CP group, the positive staining in the interstitium was abrogated completely (E). Bar = 100 μ m. $n = 6$ rats in each group. * $p < 0.05$ vs. LSP, † $p < 0.05$ vs. OHP.

sartan monotherapy. This effect may have been related to an enhancement of the reduction of proteinuria by the combination therapy. Proteinuria is known to enhance the development of tubulointerstitial injury in patients with chronic kidney disease (39). In addition to their anti-proteinuric effects, TZDs have an anti-fibrotic effect in the kidneys, as demonstrated by both *in vivo* (40) and *in vitro* (41, 42) studies. Moreover, TZDs are reported to have anti-inflammatory effects in the kidneys (40) and blood vessels (43, 44). In the present study, candesartan sufficiently suppressed monocyte/macrophage infiltration into the tubulointerstitium of the kidneys of OHP rats. Therefore, any anti-inflammatory action of the combination therapy could be ruled out based on the findings of the present study.

The limitation of the present study is that it could not be determined whether the beneficial effects of pioglitazone were BP-dependent or -independent. Concerning candesartan monotherapy, we consider that the amelioration of glomerulosclerosis is BP-dependent and the amelioration of podocyte injury, interstitial fibrosis and monocyte/macrophage infiltration is BP-independent. However, we were unable to clarify whether the effects of pioglitazone were BP-dependent or -independent, because there was no pioglitazone monotherapy group. In our previous study using the same model, pioglitazone reduced proteinuria independently of BP (45). We also consider that the additive effect of pioglitazone on candesartan was due to a reduction in proteinuria and possibly a reduction in interstitial fibrosis. However, it is unclear whether these effects were BP-dependent or -independent in this study, because OHP+CP rats showed significantly decreased SBP compared with OHP+C rats. Thus the benefits of combination therapy compared to pioglitazone monotherapy will need to be validated in the future.

In conclusion, we demonstrated in the present study that combination therapy with candesartan and pioglitazone was more effective in reducing BP and proteinuria and possibly in preventing interstitial fibrosis in the kidney, compared with candesartan monotherapy, in Zucker obese rats fed a high-protein diet. Clinically, these findings suggest that combination therapy with an ARB and a TZD may halt the development of CKD in metabolic syndrome. The detailed mechanisms of the antihypertensive, anti-proteinuric and anti-fibrotic actions of such combination therapy remain to be elucidated. Further experimental and clinical studies are needed to determine whether ARB plus TZD combination therapy is beneficial in preventing the development of CVD and CKD in patients with the metabolic syndrome.

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