Tempol or candesartan prevents high-fat diet-induced hypertension and renal damage in spontaneously hypertensive rats

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Abstract

Background. Obesity has been strongly associated with the development and aggravation of hypertension and chronic kidney disease. To date, the systemic renin–angiotensin system (RAS) has been known to involve in obesity-induced tissue damage and hypertension. However, the intrarenal mechanism whereby obesity induces and aggravates hypertension and renal disease remains poorly understood. Therefore, we investigated the role of intrarenal RAS and oxidative stress in diet-induced hypertension and renal inflammation in spontaneously hypertensive rats (SHR) fed a high-fat diet.

Methods. Male SHR and Wistar-Kyoto rats (WKY) were divided into eight groups: normal-fat diet-fed WKY (WKY-NF), high-fat diet-fed WKY (WKY-HF), high-fat diet-fed tempol-treated WKY (WKY-HF/T), high-fat diet-fed candesartan-treated WKY (WKY-HF/C), normal-fat diet-fed SHR (SHR-NF), high-fat diet-fed SHR (SHR-HF), high-fat diet-fed tempol-treated SHR (SHR-HF/T) and high-fat diet-fed candesartan-treated SHR (SHR-HF/C). After 12 weeks of treatment, haemodynamic measurements and histological assessment of the kidney were performed.

Results. At the end of week 12, the high-fat fed SHR gained more body weight, their systolic blood pressure was further elevated and glucose intolerance induced. There was no significant difference in the insulin resistance index, serum lipid profile, plasma renin activity and serum aldosterone levels according to diet. However, the high-fat diet resulted in increases in immunohistochemical stains of renin and angiotensin II in the kidney. The real-time PCR also demonstrated significant increases in mRNA levels of renin, angiotensinogen and angiotensin-converting enzyme in the kidney, reflecting enhanced activation of the intrarenal RAS, which findings were also shown by Western blot analysis for renin and angiotensin II type 1 receptor. The expression of ED-1, osteopontin and TGF-β1 in the renal cortex were prominently enhanced in the SHR-HF group with the increased intrarenal lipid concentrations and oxidative stress. Administration of tempol or candesartan in the high-fat diet-induced SHR inhibited the elevation of the systolic blood pressure, intrarenal lipid concentrations, oxidative stress and the degree of renal inflammation to the levels of, or more than, the SHR-NF with no differences in the body weight and periepididymal fat weight, compared to those in the SHR-HF group without such treatment.

Conclusions. Our study suggests that a high-fat diet induces fatty kidneys, aggravation of blood pressure and renal inflammation in the SHR. Blockade of oxidative stress by tempol or of RAS by candesartan ameliorates the increase in blood pressure and renal inflammation and improves intrarenal lipid accumulation. Therefore, antioxidants or angiotensin receptor blockers can prevent diet-induced hypertension and renal inflammation in hypertensive rats.

Keywords: hypertension; lipotoxicity; obesity; oxidative stress; renin–angiotensin system

Introduction

Obesity, in particular the visceral type of adiposity, is characterized by dysfunctional adipose tissue and is an important component of the metabolic syndrome [1,2]. Both obesity and the metabolic syndrome are associated with a high morbidity and mortality; obese individuals are at increased risk for diabetes, hypertension and other cardiovascular diseases [3]. Obesity by itself possibly accounts for 78% and 65% of essential hypertension in men and women, respectively, according to the data from the Framingham Cohort [4,5]. The prevalence of obesity worldwide is increasing and the prevalence of hypertension in individuals with obesity is also substantially elevated [3]. As well, hypertension, overweight, obesity and the metabolic syndrome have emerged as strong independent risk factors for

chronic kidney disease (CKD) and end-stage renal disease (ESRD) [6,7]; hypertensive patients account for 25% of all CKD patients, and obese patients with hypertension are at the greatest risk for developing CKD [6,8].

Clinical and experimental studies have confirmed a strong association between obesity and hypertension. However, the exact mechanism whereby obesity causes hypertension remains unknown because obesity-associated hypertension is complex, multifactorial and lacks a suitable animal model for detailed study. Currently, the main mechanisms that could explain the development of high arterial pressure in obesity include activation of the renin–angiotensin system (RAS) [9,10]. Other probable factors are the increased asymmetric dimethylarginine concentrations and increased vascular tone created by a reduced bioavailability of nitric oxide (NO) because of increased oxidative stress [11,12]. Free fatty acid (FFA) overload may also enhance production of the reactive oxygen species (ROS) [13], and high levels of circulating FFA and ROS may be involved in the pathogenesis of hypertension. Apart from these systemic derangements, there is limited information regarding the possible intrarenal alterations in obesity-induced hypertension.

The development of obesity may lead to significant lipid deposits around or within non-adipose tissues and organs, and this could impair tissue and organ function. The kidneys of obese individuals are tightly encapsulated by fatty tissue; the fat penetrates the renal hilum into the sinuses surrounding the medulla [14]. In addition to these structural changes, local accumulation of lipids may occur in non-adipose tissues such as the kidneys, leading to cell dysfunction or cell death, known as lipotoxicity [4,13,15]. Adipose tissue possesses a local RAS, with more significant local paracrine as well as systemic effects than the subcutaneous fat; this contributes to the cardiometabolic derangements including sodium retention and volume expansion, progressive renal disease and elevated blood pressure (BP) [16]. However, effects of lipid accumulation in the non-adipose kidney, one of the key organs of cardiovascular control, have been little described. Furthermore, the mechanisms associated with obesity, renal lipotoxicity, oxidative stress, RAS and hypertension have not yet been studied in detail in kidneys associated with fat accumulation.

In the present study, we investigated the effects of a high-fat diet (HFD) on the kidney, BP and intrarenal morphology. The intrarenal lipid accumulation that develops from 12 weeks of high-fat feeding, in spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY), might contribute to the renal and systemic changes via intrarenal oxidative stress and RAS activation. Therefore, to determine whether oxidative stress and RAS, in HFD-induced obese rats, had direct effects on BP and renal injury, an angiotensin receptor blocker (ARB) candesartan (Cand) or a superoxide dismutase (SOD) mimetic tempol was given during the same period.

Subjects and methods

Animals and experimental design

Seven-week-old male SHR weighing 220 ± 5 g were purchased from Japan SLC (Nakaizu, Japan), and WKY were used as controls in all experiments.

The rats were fed either a normal-fat or a HFD for 12 weeks starting at the age of 8 weeks. The rats were divided into eight groups: WKY fed a standard rat chow (WKY-NF, $n = 6$), WKY fed a HFD (WKY-HF, $n = 6$), WKY fed a HFD and given 20 µg/kg/day of tempol (Sigma-Aldrich, Steinheim, Germany) in drinking water (WKY-HF/T, $n = 6$), WKY fed a HFD and given 10 µg/kg/day of candesartan cilexetil (AstraZeneca, Mölndal, Sweden) in the drinking water (WKY-HF/C, $n = 6$), SHR fed a standard rat chow (SHR-NF, $n = 6$), SHR fed a HFD (SHR-HF, $n = 8$), SHR fed a HFD and given 20 µg/kg/day of tempol in drinking water (SHR-HF/T, $n = 8$), and SHR fed a HFD and given 10 µg/kg/day of candesartan cilexetil in the drinking water (SHR-HF/C, $n = 8$). The standard rat chow (Samyang, Wonju, Korea) consisted of 16% Kcal fat, 58% Kcal carbohydrate, 26% Kcal protein, 25% Kcal soybean oil and 0.23% NaCl. The high-fat diet (#D12451 Research Diet, NJ, USA) consisted of 45% Kcal fat, 35% Kcal carbohydrate, 20% Kcal protein, 25% Kcal soybean oil and 0.23% NaCl. The experimental protocols were approved by the Animal Care Committee of The Catholic University of Korea.

Physiological and biochemical measurements

The rat body weight and systolic BP were measured every 4 weeks. BP was assessed under conscious conditions using the tail-cuff method with an electrosphygnomanometer (IITC Life Science, Woodland Hills, CA, USA). Blood glucose levels were measured with an Accu-check meter (Roche diagnostics, St Louis, MO, USA) and glycosylated haemoglobin (HbA1c) was determined by an autoanalyser (Bayer Healthcare, LLC, IN, USA). The serum total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol and free fatty acid (FFA) concentrations were measured by an autoanalyser (Wako, Osaka, Japan). Plasma renin activity (Renin-RIA BEAD, SRL, Tokyo, Japan) and serum aldosterone levels (Diagnostic Systems Laboratories, Webster, TX, USA) were measured using an RIA kit. The intraperitoneal glucose tolerance and HbA1c were measured at the end of the study. Plasma insulin concentrations were measured by RIA (Wako, Osaka, Japan), and homeostasis model assessment for the insulin resistance (HOMA-IR) index was calculated as follows: fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5 [17]. For measurement of the 24 h urine volume, urinary albumin excretion, urine chemistry and creatinine clearance, the rats were placed in individual rat metabolic cages (Tecniplast, Gazzada, Italy) with access to water and food for 24 h. The 24-h urinary albumin excretion was measured by immunoassay (Bayer, Elkhart, IN, USA). At week 12, plasma and urinary creatinine concentrations were measured using an autoanalyser (Beckman Instruments, Fullerton, CA, USA) and the creatinine clearance was calculated. The plasma and urinary sodium, potassium and osmolality were measured by the ADVIA 1650 System (Bayer, Tarytown, NY, USA) and the fractional excretion of sodium (FeNa) and transtubular potassium gradient (TTKG) were calculated as follows: FeNa (%) = (urinary sodium × plasma creatinine)/(plasma sodium × urinary creatinine) × 100, TTKG = urinary potassium/(plasma osmolality/urinary osmolality)/plasma potassium. To evaluate the RAS activity and oxidative stress, we measured the 24-h urinary aldosterone and 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentrations. Nitrate/nitrite was also assayed in plasma and urine by use of an LDH colorimetric method.

Histological assessment

After 12 weeks, all rats were sacrificed, and immunohistochemical analysis of the kidneys was performed. The histology was assessed after periodic acid-Schiff (PAS) staining. The mesangial matrix area and glomerular tuft area were quantified for each glomerular cross-section as previously reported [18,19]. More than 30 glomeruli that were cut through the vascular pole were counted per kidney, and the average was used for the analysis. We performed immunohistochemistry for renin, angiotensin II (Ang II), osteopontin, ED-1, TGF-β1 and 8-OHdG. Kidney sections were incubated overnight with anti-renin (1:8000; provided by Dr Kirsten Madsen, Department of Medicine, University of Florida, Gainesville, FL, USA), anti-Ang-II (1:200; Peninsula Labs Inc., San Carlos, CA, USA), anti-ED-1 (1:1000; Serotec Inc., Raleigh, NC, USA), anti-osteopontin (1:2000; MPIIIB10, obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), anti-TGF-β1 (1:100; SantaCruz Biotechnology, Santa Cruz, CA, USA) and anti-8-OHdG (1:100; JalCA, Shizuoka, Japan) in a humidified chamber at 4°C. All of these sections were examined in a blinded manner using light microscopy (Olympus BX-50; Olympus Optical, Tokyo, Japan). For the quantification of the proportional area

Table 1. Overall body weight gain, periepididymal fat weight, serum lipid profiles, HbA1c, plasma insulin level, HOMA-IR after 12 weeks

|	WKY-NF	WKY-HF	WKY-HF/T	WKY-HF/C	SHR-NF	SHR-HF	SHR-HF/T	SHR-HF/C
Weight gain (%)	200	208[a]	205	194	170	185[b]	203[b]	206[b]
Periepididymal fat weight (g)	4.0 ± 0.4	5.9 ± 1.0[c]	5.3 ± 0.4[c]	4.5 ± 0.5[c]	2.5 ± 0.2	5.2 ± 0.9[c]	5.4 ± 0.6[c]	5.3 ± 0.4[c]
Total cholesterol (mg/dL)	127.2 ± 3.5	118.3 ± 15.8	114.8 ± 14.1	106.7 ± 1.5	69.8 ± 4.6[d]	71.9 ± 7.0[d]	77.9 ± 7.8[d]	82.1 ± 5.7[d]
Triglyceride (mg/dL)	29.0 ± 4.0	39.0 ± 14.4	21.5 ± 14.0	21.7 ± 3.8	40.0 ± 12.7	30.1 ± 9.6	36.1 ± 15.1	36.3 ± 14.6
LDL-cholesterol (mg/dL)	17.8 ± 1.0[e]	17.3 ± 2.2[e]	9.3 ± 2.6	6.3 ± 0.6	9.8 ± 0.8	11.3 ± 1.0	9.1 ± 2.2	9.5 ± 1.1
HDL-cholesterol (mg/dL)	41.8 ± 1.2	37.0 ± 5.2	33.5 ± 5.2	33.3 ± .25	29.2 ± 2.0[d]	25.1 ± 1.6[d]	24.3 ± 1.7[d]	27.1 ± 1.6[d]
Free fatty acid (µEq/L)	442.8 ± 72.5	471.0 ± 93.3	327.3 ± 10.5	382.5 ± 108.2	422.7 ± 116.9	636.3 ± 61.7[f]	459.9 ± 94.1	453.5 ± 65.8
HbA1c (%)	3.6 ± 0.1	3.4 ± 0.1	3.5 ± 0.1	3.4 ± 0.2	3.5 ± 0.2	3.4 ± 0.3	3.5 ± 0.1	3.6 ± 0.1
Insulin (pg/L)	2.6 ± 1.6	2.5 ± 0.9	1.5 ± 0.2	1.3 ± 0.2	1.2 ± 0.8	1.5 ± 0.4	1.3 ± 0.4	1.2 ± 0.5
HOMA-IR	0.75 ± 0.46	0.67 ± 0.22	0.50 ± 0.30	0.49 ± 0.25	0.46 ± 0.34	0.55 ± 0.20	0.66 ± 0.15	0.58 ± 0.25

Values are expressed as mean ± SD. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; Weight gain, overall weight gain percentage when compared with the initial weight; HbA1c, glycosylated haemoglobin; HOMA-IR, homeostasis model assessment for insulin resistance.
[a]$P < 0.05$ versus WKY-NF and WKY-HF/C; [b]$P < 0.05$ versus SHR-NF; [c]$P < 0.001$ versus WKY-NF and SHR-NF; [d]$P < 0.05$ versus WKY groups; [e]$P < 0.05$ versus other groups; [f]$P < 0.01$ versus other groups.

of staining, ∼20 views (×400 magnification) were randomly located in the renal cortex and corticomedullary junction of each slide. As previously described [20], Scion Image software v. Beta 4.0.2 (Scion corp., Frederick, ML, USA) was used to semi-automatically estimate the volume fraction of immunopositive cells within the tissue sample. The kidney lipid content was measured using an assay kit from Waco Co. (Osaka, Japan). Renal parenchymal lipids were extracted by the method of Bligh and Dyer with slight modifications [21]. A portion (200 mg) of the kidney was homogenized and extracted by methanol-chloroform aliquots (2:1) in a 37°C water bath under N_2. The lower chloroform phase was withdrawn, and the lipids were measured using the assay kits. The renal tissue Ang II concentration was also determined by a modification of a previously described method [22]. For RNA isolation and quantification of gene expression, total RNA was extracted from the kidney tissue using RNA-Bee (TEL-TEST, Texas, TX, USA) and cDNA was synthesized from 2 µg of total RNA with random hexamers and the AccuPower Cycle Script (Bioneer, Daejeon, Korea). Real-time PCR was performed on the MX-3000P real-time detection system (Stratagene, La Jolla, CA, USA) using SYBR premix Ex Taq (Takara, Otsu, Japan). The 18S rRNA was used as an endogenous control gene. Primers used for angiotensinogen (AGT), renin and angiotensin-converting enzyme (ACE) were as follows: for AGT, 5′-CACGGACAGCACCCTATTTT-3′ and 5′-GCTGTTGTCCACCCAGAACT-3′, for 18S rRNA, 5′-CGCGG TTCTATTTTGTTGGT and 5′-AGTCGGCATCGTTTATGGTC-3′, for renin, 5′-TCTCTCCCAGAGGGTGCTA-3′ and 5′-CCCTCCTCA CACAACAAGGT-3′, for ACE, 5′-AGCCATCCTTCCCTTTTTC-3′ and 5′-GGCTGCAGCTCCTGGTATAG-3′. The PCR products of AGT, renin and ACE were amplified on the Gene Amp PCR system 9700 (PE applied Biosystem, Foster city, CA, USA) and analysed on a 1% agarose gel, normalized against the density of the 18S rRNA, using Multi gauge version 3.0 (Fuji Film, Tokyo, Japan). The expression of Ang II type 1 (AT_1) receptor and renin in the kidney cortex of various rat groups was determined by Western blotting. For this purpose, the kidney cortices were homogenized in the buffer containing (in mmol/L): Tris 50, EDTA 10, PMSF 1 and a mixture of protease inhibitors (aprotinin, calpain inhibitors, leupeptin, pepstatin and trypsin inhibitor). Proteins in the homogenates were determined by the BCA method using a kit. Equal amounts of protein (30 µg for AT_1 and 30 µg for renin) from various rat groups were subjected to SDS-PAGE and electroblotting onto Immobilon P (blot). The blot was incubated with primary polyclonal antibodies for the AT_1 receptor or renin. After the incubation with the primary antibodies, the blots were incubated with horseradish peroxidase-conjugated antirabbit IgGs. The signal was detected by an enhanced chemiluminescence system and recorded and analysed for the densitometry of the bands. For loading control, the blots were stripped and reprobed with a β-actin antibody.

Statistical analyses

Data were expressed as the mean ± SD. Statistical differences between the groups were determined by two-way ANOVA with the Bonferroni correction. Non-normally distributed data were analysed by the nonpara-
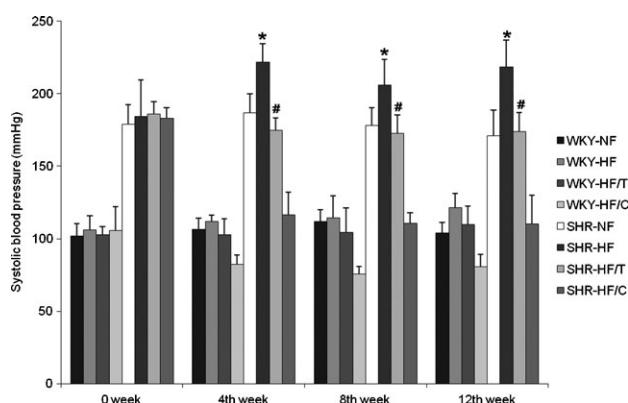
Fig. 1. Systolic blood pressure throughout the experiment. Blood pressure of SHR-HF/T was significantly increased compared with other groups beginning during the fourth week of the diet. *$P < 0.001$ versus other groups; #$P < 0.001$ versus WKY groups and SHR-HF/C.

metric Mann–Whitney test. A P-value <0.05 was considered a statistically significant difference.

Results

Body weight and systolic BP

After 12 weeks, the WKY-HF gained modest body weight compared to the WKY-NF and WKY-HF/C, and the SHR-HF, SHR-HF/T and SHR-HF/C weighed more than the SHR-NF ($P < 0.05$, Table 1). The harvested periepididymal fat weights of the HFD-fed WKY and SHR groups increased more than the normal-fat diet-fed WKY-NF and SHR-NF ($P < 0.01$). The systolic BP of the SHR-NF and the SHR-HF groups were higher throughout the entire experimental period than the WKY-NF and the WKY-HF groups ($P < 0.001$, Figure 1). The systolic BP of the SHR-HF/T was higher than the WKY-NF and WKY-HF, lower than the SHR-HF ($P < 0.001$), and was similar to that of the SHR-NF. As expected, the systolic BP was even more decreased in the SHR-HF/C, compared to the SHR-NF ($P < 0.001$). The systolic BP tended to be higher in the WKY-HF, compared to the WKY-NF; however, the difference was not

Table 2. Plasma renin activity, aldosterone and renal function parameters after 12 weeks

	WKY-NF	WKY-HF	WKY-HF/T	WKY-HF/C	SHR-NF	SHR-HF	SHR-HF/T	SHR-HF/C
Serum creatinine (mg/dL)	0.66 ± 0.05	0.70 ± 0.06	0.68 ± 0.03	0.69 ± 0.05	0.66 ± 0.05	0.62 ± 0.05	0.66 ± 0.05	0.67 ± 0.06
PRA (ng/mL/h)	7.6 ± 6.90	8.82 ± 5.38	6.11 ± 1.12	9.07 ± 6.84	7.96 ± 5.47	7.74 ± 4.98	7.85 ± 5.90	9.30 ± 5.90
Aldosterone (pg/mL)	285.9 ± 73.2	304.9 ± 108.6	361.5 ± 125.3	308.4 ± 98.3	277.8 ± 95.4	305.0 ± 111.4	277.1 ± 77.9	267.2 ± 54.3
24-h urine volume (mL/day)	27.3 ± 13.4	27.7 ± 4.6	25.3 ± 4.6	27.3 ± 4.6	17.2 ± 2.1	12.6 ± 3.9[a]	16.3 ± 7.3	14.3 ± 1.5
24-h urine albumin (mg/mL/day)	3.31 ± 0.58	2.80 ± 0.67	0.53 ± 0.27[b]	0.19 ± 0.05[b]	3.57 ± 1.10	2.92 ± 0.95	2.34 ± 1.68	2.50 ± 0.03
Creatinine clearance (mL/min 100 g body weight)	0.53 ± 0.25[b]	0.77 ± 0.14[b]	0.33 ± 0.05	0.30 ± 0.04	0.30 ± 0.04	0.28 ± 0.03	0.22 ± 0.04	0.29 ± 0.02
FeNa (%)	0.41 ± 0.12	0.26 ± 0.07	0.27 ± 0.11	0.49 ± 0.06	0.55 ± 0.21	0.20 ± 0.07[b]	0.36 ± 0.09	0.34 ± 0.08
TTKG	7.33 ± 0.52	5.83 ± 0.41	6.53 ± 0.86	7.87 ± 1.24	6.17 ± 0.98	8.13 ± 0.64[b]	5.62 ± 1.06	5.4 ± 0.89

Values are expressed as mean ± SD. PRA, plasma rennin activity; FeNa, fractional excretion of sodium; TTKG, transtubular potassium gradient.
[a]$P < 0.01$ versus WKY groups; [b]$P < 0.05$ versus other groups.

statistically significant. The systolic BP of the WKY-HF/T was not changed, whereas the systolic BP of the WKY-HF/C tended to be lower than that of the WKY-HF ($P < 0.05$).

Biochemical data

The FFA levels in the SHR-HF were higher than in any other group ($P < 0.01$, Table 1). The serum total cholesterol and HDL-cholesterol levels were significantly lower in the SHR groups ($P < 0.05$). In the results of intraperitoneal glucose tolerance test, the SHR-HF had a higher increase in the blood glucose levels from 60 to 120 min ($P < 0.05$) than the other groups, while the SHR-HF and WKY-HF treated with tempol or Cand had lower blood glucose levels from 90 to 120 min than the WKY-NF, WKY-HF and SHR-HF ($P < 0.05$, data not shown). The serum triglyceride levels, HbA1c, insulin concentration, HOMA-IR and fasting glucose levels showed no significant difference among all groups.

Systemic RAS and renal functional parameters

There was no difference in the 24-h urine albumin excretion among the SHR groups (Table 2). Among the WKY groups, the 24-h urine albumin excretion was lowest in the WKY-HF/C. For the SHR-HF/C and WKY-HF/C, the serum aldosterone level was lower and the PRA was higher; however, this difference was not significant. The 24-h urine volume was lower in the SHR-HF than in the WKY groups ($P < 0.01$), and the creatinine clearance demonstrated higher values in the WKY-NF and WKY-HF, compared with the other groups ($P < 0.05$). The FeNa was observed to be lower in the SHR-HF. The TTKG was most increased in the SHR-HF, compared to the other groups ($P < 0.05$). In contrast, the SHR-HF/T and SHR-HF/C demonstrated reversal to the levels of FeNa and TTKG observed in the SHR-NF.

Intrarenal lipid concentration

Intrarenal concentrations of triglyceride and FFA were increased in the rats with a HFD (Figure 2A and B). The increased intrarenal lipid concentrations with the HFD in the WKY and SHR were attenuated with tempol or Cand treatment.
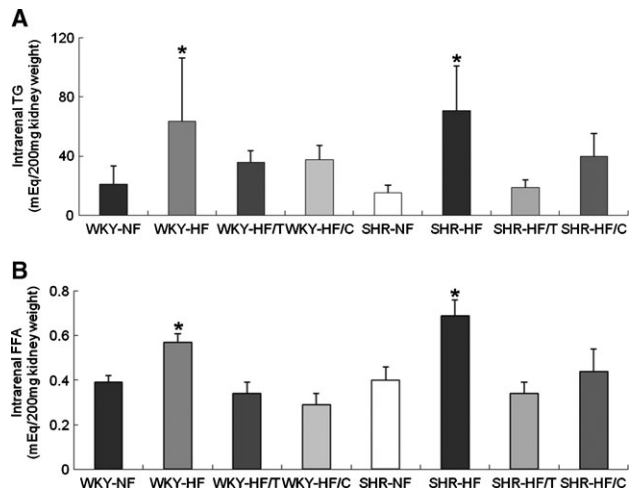
Fig. 2. Intrarenal triglyceride and free fatty acid concentrations after 12 weeks on a high-fat diet. (**A**) Intrarenal triglyceride levels were increased in rats fed a high-fat diet; these findings were attenuated with tempol or candesartan treatment in the high-fat diet-fed WKY or SHR. *$P < 0.05$ versus other groups. (**B**) Intrarenal free fatty acid concentration after 12 weeks of a high-fat diet was increased. Tempol or candesartan treatment decreased intrarenal FFA in the high-fat diet-fed WKY or SHR. *$P < 0.05$ versus other groups. TG, triglyceride; FFA, free fatty acid.

Intrarenal RAS

The HFD induced an increase in renin expression in the juxtaglomerular area of the kidney (Figures 3A and 9). In addition, the immunoreactivity and concentration of Ang II were significantly increased in the kidney of the SHR-HF (Figure 3B, C and 9). Urinary aldosterone was also significantly increased in the SHR-HF (Figure 4). Quantification of the mRNA expression by real-time PCR demonstrated increases in renin, ACE and AGT gene expression in the kidney cortex tissues from the HFD-fed SHR (Figure 5). Western blot demonstrated the presence of renin as 41 kDa band in the renal cortex (Figure 6A). Densitometric analysis of the bands suggests that the cortical renin expression in the SHR-HF was significantly higher compared with the SHR-NF. The cortical AT$_1$ receptor expression (43 kDa band) was also significantly greater in the SHR-HF compared with other groups (Figure 6B).

In contrast to the SHR-HF, tempol treatment in the SHR-HF/T resulted in a decrease in the expression of renin and
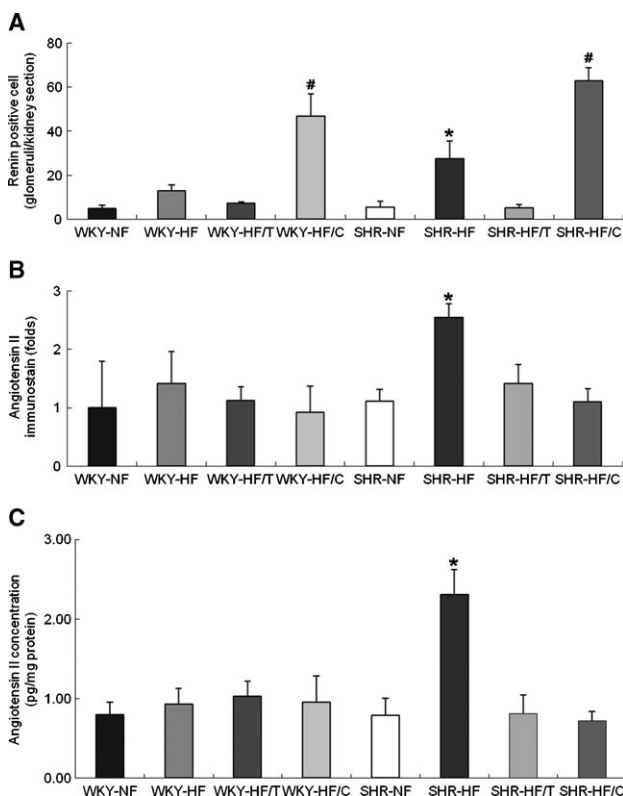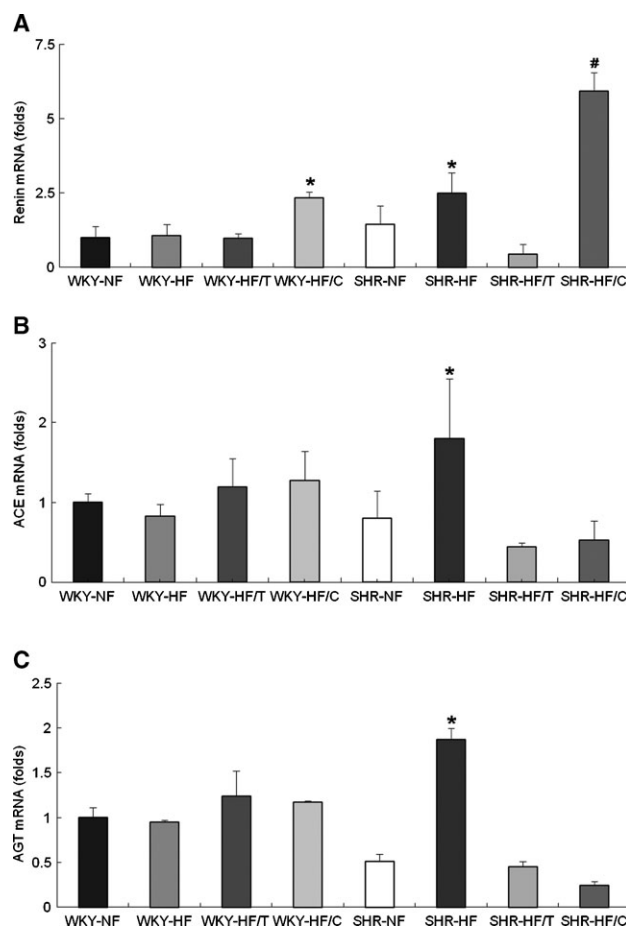
Fig. 3. Renin and angiotensin II positive glomeruli/kidney section folds after 12 weeks of a high-fat diet. (**A**) Renin expression was increased in the high-fat diet-fed rats; these findings were decreased in the tempol-treated SHR-HF/T. However, candesartan treatment in the WKY-HF/C and SHR-HF/C increased renin expression in the kidneys. *$P < 0.001$ versus WKY-NF, WKY-HF, WKY-HF/T, SHR-NF and SHR-HF/T; #$P < 0.001$ versus other groups. (**B**) The high-fat diet-fed SHR enhanced expression of angiotensin II, while tempol or candesartan treatment of the high-fat diet-fed SHR reduced these findings. *$P < 0.05$ versus other groups. (**C**) The renal tissue angiotensin II concentration in the SHR-HF was elevated in the kidney; this finding was attenuated by tempol or candesartan. *$P < 0.01$ versus other groups.
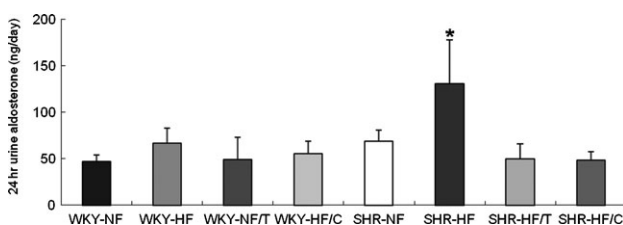
Fig. 4. The 24-h urine aldosterone levels after 12 weeks of a high-fat diet. Increased aldosterone levels after a high-fat diet in the SHR were attenuated by tempol or candesartan treatment. *$P < 0.001$ versus other groups.


Fig. 5. Quantification of mRNA expression of renin, angiotensin converting enzyme and angiotensinogen by real-time PCR in the kidneys of rats fed either a normal-fat or high-fat diet for 12 weeks. (**A**) Increased renin mRNA expression after a high-fat diet was attenuated by tempol treatment but was increased after candesartan treatment. *$P < 0.05$ versus WKY-NF, WKY-HF, WKY-HF/T, SHR-NF and SHR-HF/T; #$P < 0.001$ versus other groups. (**B** and **C**) Angiotensin-converting enzyme and angiotensinogen mRNA expression were the highest for the SHR-HF. ACE, angiotensin converting enzyme; AGT, angiotensinogen. *$P < 0.01$ versus other groups.

Ang II (Figures 3A, B, C and 9). As expected, the SHR-HF/C and WKY-HF/C had markedly increased immunoreactivity of renin in the kidney compared to the other groups (Figures 3A and 9). However, the SHR-HF/C had a significantly decreased Ang II immunoreactivity and concentration in the kidney, compared to the SHR-HF (Figures 3B, C and 9). The increased 24-h urinary aldosterone level in the SHR-HF was also attenuated by tempol or Cand treatment in SHR-HF/C and SHR-HF/T groups (Figure 4). The

renin, ACE and AGT gene expression were suppressed in the tempol-treated SHR-HF/T, compared to the SHR-HF (Figure 5A, B and C). In the Cand-treated SHR-HF/C, the renin gene expression was markedly increased and the ACE and AGT gene expression were suppressed (Figure 5A, B and C). Although the renin expression was increased in the Cand-treated SHR-HF/C, the tempol or Cand treatment of SHR fed a HFD caused a significant decrease in the cortical AT_1 receptor expression (Figure 6). These data demonstrated that the HFD induced intrarenal RAS activation in the SHR, which was inhibited by Cand or tempol treatment.

Oxidative stress in the kidneys and nitrite/nitrate content of urine

The SHR-HF had a significantly higher urinary level and immunoreactivity of 8-OHdG. However, Cand or tempol treatment in the SHR-HF/C and SHR-HF/T groups decreased urinary 8-OHdG levels and its renal
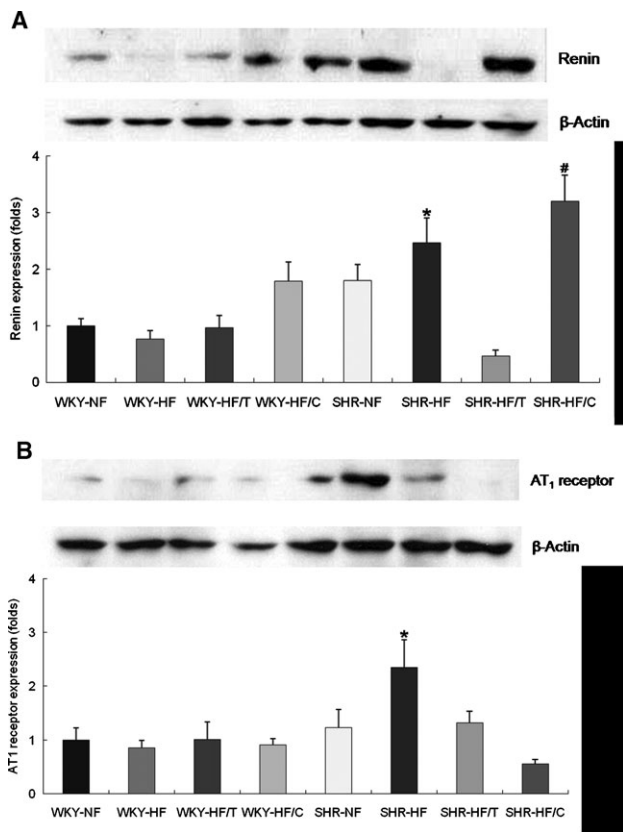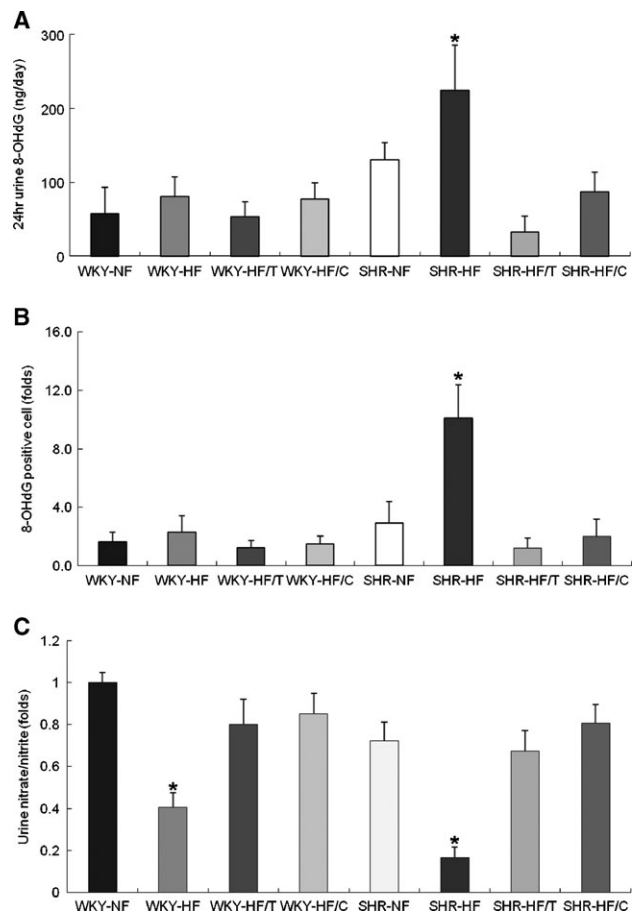
Fig. 6. Representative Western blots for renin and AT$_1$ receptor expression (top) and bar graphs of renin and AT1 receptor bands density normalized with β-actin as loading control in the kidney cortex (bottom). (**A**) Expression of renin in the SHR-HF is significantly different compared with the SHR-NF. *$P < 0.05$ versus WKY groups, SHR-NF and SHR-HF/T. Expression of renin in the Cand-treated SHR-HF/C is markedly increased. #$P < 0.05$ versus other groups. (**B**) AT$_1$ receptor expression in the SHR-HF is significantly different from other rats. *$P < 0.01$ versus other groups.

Fig. 7. The urinary concentration and renal immunoreactivity of 8-hydroxy-2′-deoxyguanosine concentration and nitrite/nitrate content of urine after 12 weeks. (**A**) The value (ng/day) in the high-fat diet-fed SHR was the highest among all groups. However, tempol or candesartan treatment decreased 8-OHdG concentration. 8-OHdG, 8-hydroxy-2′-deoxyguanosine. *$P < 0.001$ versus other groups. (**B**) Intrarenal 8-OHdG immunoreactivity was increased in the kidneys of SHR-HF; this finding was attenuated by tempol or candesartan. *$P < 0.01$ versus other groups. (**C**) Urine nitrite/nitrate content (folds) was decreased in SHR or WKY fed a HFD; this finding was improved by tempol or candesartan. *$P < 0.01$ versus other groups.

immunoreactivity (Figure 7A and B). Figure 7C illustrates a decrease in urine nitrite/nitrate content of SHR or WKY fed a HFD, indicating a decreased production or bioavailability of NO compared with SHR or WKY fed a normal chow. By contrast, tempol or Cand improved the urine nitrite/nitrate content. There were no differences in the plasma nitrite/nitrate contents among all groups (data not shown).

Renal injury markers and renal histology

Quantitative immunohistochemical analysis for osteopontin showed that the expression of osteopontin in the renal cortex was more pronounced in the SHR-HF compared to the SHR-NF (Figures 8A and 9). The number of ED-1 positive cells was 2- to 3-fold greater in the intra- and periglomerular area of the SHR-HF compared to the SHR-NF (Figures 8B and 9). Immunohistochemical analysis for TGF-β1 indicated that the kidneys of the WKY-HF and SHR-HF exhibited more TGF-β1 than those of the WKY-NF and SHR-NF (Figures 8C and 9). Analysis of PAS-stained kidney sections revealed that there was a marked expansion of the mesangial area in the SHR-HF compared

to the SHR-NF (Figures 8D and 9). These changes in renal morphology and inflammation were more prominent in the SHR-HF than in the WKY-HF, and such findings in the SHR-HF were attenuated in Cand- or tempol-treated SHR-HF/C and SHR-HF/T.

Discussion

All rats with a high-fat intake in the present study became obese, reflecting an increase in the adipose mass as measured by the periepididymal fat weight. With prolonged obesity, the HFD affected blood pressure and the renal histology. One of the most important findings of this study was that the SHR fed a HFD aggravated their hypertension. Several studies have addressed the issue whether a HFD increases BP in the SHR. One study observed that a HFD
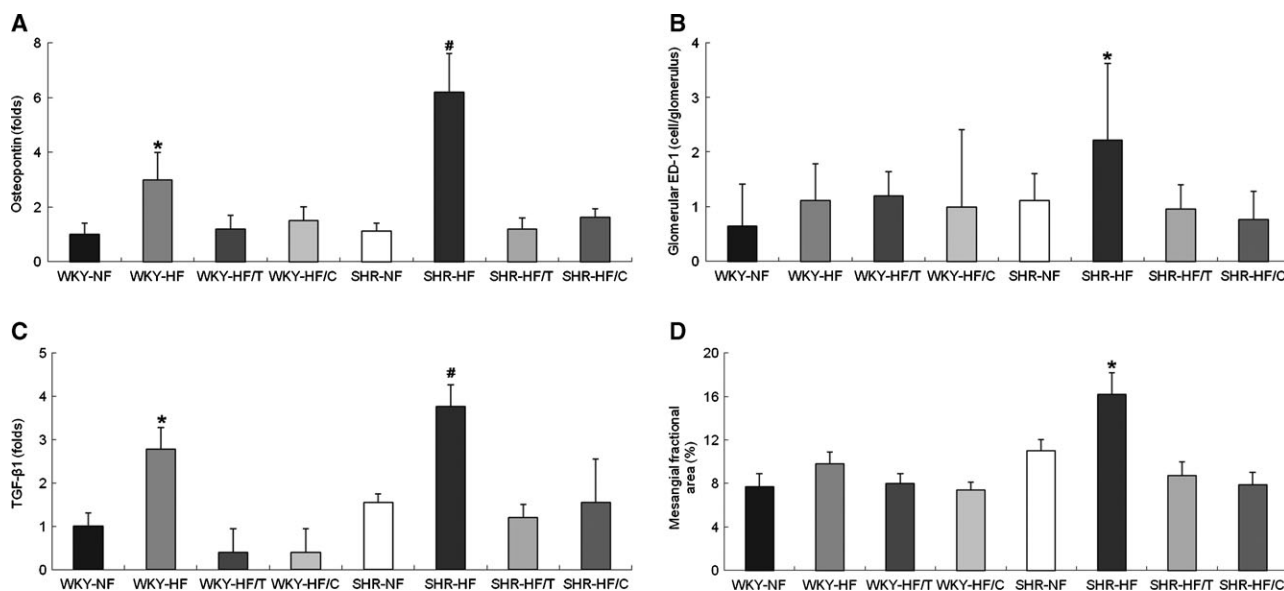
Fig. 8. Intrarenal immunoreactivity of osteopontin (**A**), ED-1 positive cells (glomerular area; B), TGF-β1 (**C**) and mesangial fractional area (**D**) after 12 weeks of a high-fat diet. (**A**) *$P < 0.05$ versus other groups; #$P < 0.01$ versus other groups. (**B**) *$P < 0.05$ versus other groups. (**C**) *$P < 0.05$ versus WKY-NF, WKY-HF/T, WKY-HF/C, SHR-NF, SHR-HF/T and SHR-HF/C; #$P < 0.01$ versus WKY-NF, WKY-HF/T, WKY-HF/C, SHR-NF, SHR-HF/T and SHR-HF/C. (**D**) *$P < 0.01$ versus other groups.

for 10 weeks had no effect on systolic blood pressure in the SHR [23], while another study using 24-h radiotelemetry measurements showed that dietary obesity in the SHR fed a HFD for 12 weeks delayed BP and HR recovery from stress and increased BP and HR during the night, when rats, as nocturnal animals, were behaviorally active [24]. Because BP was measured in the conscious state, which was a kind of stressful condition, we found that a HFD could increase BP in the SHR. A significant increase of BP in the SHR-HF that exceeded the normal threshold of the control SHR was noted even after 4 weeks of the HFD; this effect lasted throughout the experiment without systemic RAS activation, significant insulin resistance or definite dyslipidaemia. RAS is important in blood pressure control; results from experimental and human studies suggest activation of the RAS with obesity and hypertension [25]. There is a great deal of evidence supporting the activation of the RAS in association with obesity [3]. Increased serum aldosterone levels appear to be linked to the development of obesity-related hypertension [26]. Moreover, diverse clinical studies have observed elevated plasma aldosterone levels in obese patients [27–31]. In this study, however, there was no clear difference in the PRA and the plasma aldosterone levels among the groups, according to diet. The increase in the systolic BP was not correlated with the PRA or plasma aldosterone levels; these findings suggest that the HFD for 12 weeks made no significant difference in the PRA and plasma aldosterone levels. The link between obesity and increased aldosterone secretion remains unclear, and the implications of the systemic RAS, in obesity-related hypertension, needs to be defined more clearly [32]. On the other hand, it is noteworthy that all components of the RAS are present in the kidney and that the intrarenal RAS operates independently of the factors that regulate the plasma RAS

activity [33,34]. The results of the present study showed that the RAS in the kidneys of the HFD-induced SHR was significantly enhanced, with increased renal tubular sodium reabsorption and aldosterone action. This sodium retention is consistent with earlier reports using dogs or rabbits [35]. In addition, the mRNA expression of renin, ACE and AGT was increased in kidneys of the SHR-HF group, whose results were also supported by Western blot analysis for renin and AT$_1$ receptor. However, Cand or tempol treatment in the SHR fed a HFD attenuated the findings associated with RAS activation. Taken together, in this rat HFD-induced obesity model, the aggravated hypertension was most likely caused by intrarenal, rather than systemic, RAS activation.

This intrarenal RAS activation appears to be correlated with increased lipid accumulation and oxidative stress in the kidney. There has been no detectable fat accumulation by extraction procedures within the renal parenchyma in previous reports [35]. In contrast, the HFD-fed rats in this study showed increased lipid accumulation in the renal parenchyma. This ectopic fat could act as a secretory organ to influence the involved tissue. In general, adipose tissue has been shown to express most of the proinflammatory mediators and to increase oxidative stress [1]. Considering that there is a countervailing interaction between endothelial NO and Ang II [36], the balance between NO, Ang II and vascular generation of ROS appear to be crucial for maintaining the homeostasis of the cardiovascular and renal systems, and during the pathogenesis of hypertension in both animal models and humans; this balance becomes perturbed so that the actions of Ang II predominate over those of NO, or inhibition of NO production stimulates generation of Ang II and superoxide anions [37]. The reduction in NO bioavailability may also explain the increased renin
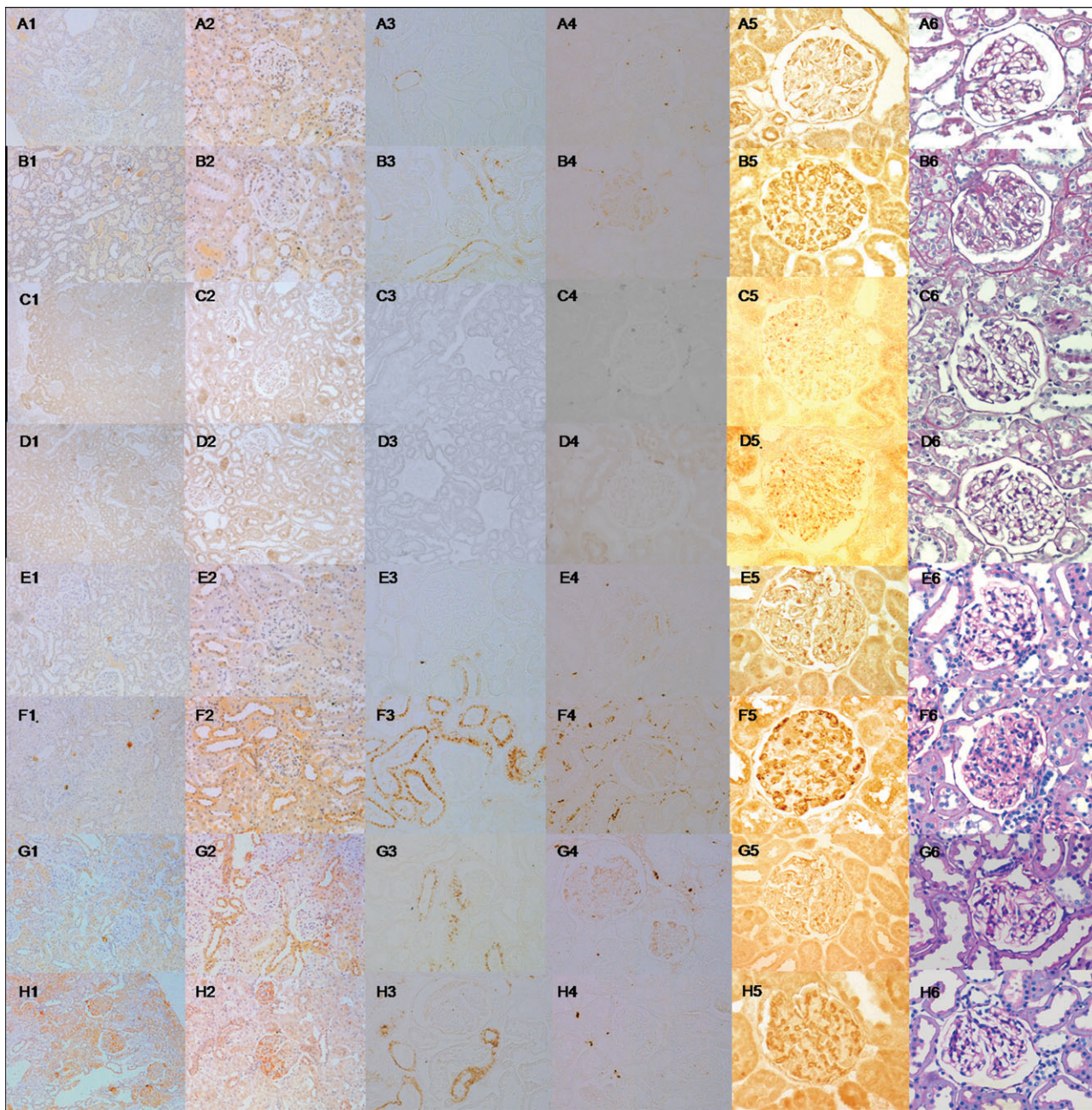
Fig. 9. Immunohistochemistry for renin, angiotensin II, osteopontin, ED-1, and TGF-β1 and PAS stain for glomeruli in the experimental animals. Representative immunostained findings at 12 weeks after a high-fat diet for renin (A1-H1), angiotensin II (A2-H2), osteopontin (A3-H3), ED-1 (A4-H4), TGF-β1 (A5-H5), and PAS-stained glomeruli (A6-H6). WKY-NF (A1-A6), WKY-HF (B1-B6), WKY-HF/T (C1-C6), WKY-HF/C (D1-D6), SHR-NF (E1-E6), SHR-HF (F1-F6), SHR-HF/T (G1-G6), and SHR-HF/C (H1-H6) after 12 weeks. Original magnification ×100 for renin; ×200 for angiotensin II and osteopontin; ×400 for ED-1, TGF-β1 and PAS.

activity in HFD-induced SHR, as stated previously [38]. The results of the present study showed that reducing the oxidative stress with an SOD mimetic tempol or blocking RAS by the ARB Cand lowered BP in the SHR fed a HFD. These results emphasize that increasing oxidative stress or RAS activation, which may be associated with intrarenal lipid accumulation, plays an important role in the pathogenesis of obesity-associated hypertension. Increasing oxidative stress was reported to elevate BP further in

the SHR but not in the WKY, suggesting that the WKY were protected due to the higher bioavailable levels of NO, and the ability to upregulate catalase and glutathione peroxidase [39]. This explains the differences of the response to diet between the two strains.

Interestingly, we observed that treatment with Cand or tempol inhibited lipid accumulation only in the kidneys of the SHR fed a HFD, without changes in the total body weight and periepididymal fat weight. The precise effects

of Cand or tempol on lipid accumulation in the kidneys of SHR fed a HFD are unknown. In type 2 diabetes, renal accumulation of fatty acids and cholesteryl esters occurs concurrently with progression of tubulointerstitial fibrosis and glomerulosclerosis, increased production of TGF-β, Ang II and albuminuria [40]. Ang II has been shown to increase the sterol regulatory element-binding protein-1 (SREBP-1) expression and lipid accumulation in renal tubular and vascular wall cells [4]. In a rat model, the pharmacological inhibition of FFA β-oxidation led to intramyocellular lipid accumulation, which was exacerbated in the setting of a HFD [15], and the administration of an ACE inhibitor resulted in the reduction of ROS-mediated lipid peroxidation, providing protective mechanisms in the kidneys [41]. Therefore, strategies that divert excess lipid away from adipose kidney tissues are likely to decrease lipotoxicity. Administration of a peroxisome proliferator activated receptor (PPAR) γ prevented diabetes and cardiomyopathy by decreasing ectopic deposition of fat and presumably increasing adipose tissue accumulation [15,42]. ARB might attenuate the lipid accumulation in the kidneys via the same effects of RAS inhibition such as ACE inhibitors or direct activation of PPARγ [43,44]. Tempol treatment would also reduce intrarenal oxidative stress, sequentially block the intrarenal RAS activation and then decrease intrarenal fat accumulation. Future studies are warranted to identify the mechanisms involved in the lipid-lowering effects of ARB or SOD mimetic in fatty kidneys.

Hypertension and obesity were accompanied by changes in the renal morphology and inflammation. Consistent with the findings that anomalous inflammatory responses, triggered by the metabolic syndrome, caused renal injury and linked renal lipid accumulation with lipotoxicity to inflammation [45,46], there were also increases in the mesangial matrix, TGF-β1 expression and inflammatory cell infiltration in the kidneys of rats exposed to a HFD; these findings improved with Cand or tempol treatment. As several investigators have stated previously, ROS and Ang II appear to participate in TGF-β expression, and TGF-β promotes fibrotic changes that lead to renal damage [47,48].

In contrast to many other models of diet-induced obesity [38,49], there was no significant dyslipidaemia observed during this experiment after 12 weeks of the diet. The total cholesterol, LDL-cholesterol and HDL-cholesterol were significantly increased in the WKY rats only, and the triglyceride levels showed no significant differences among strains. Dyslipidaemia, which occurs in other models of diet-induced obesity, is a common feature in humans and is frequently associated with hypertension [50]. There might be a difference between the two strains, SHR and WKY, in the clearance of cholesterol. Along with high-fat feeding, the postprandial glucose tolerance deteriorated without serious impairment of the secretion and sensitivity of insulin. Hyperinsulinaemia and insulin resistance have been postulated to be responsible for arterial hypertension in obese human beings [3,9,51], while other studies have suggested that hyperinsulinaemia does not necessarily lead to hypertension in obese people [52]. The present results did not demonstrate that a high-fat intake for 12 weeks induced a definite diabetic state in the rats. In prospectively observed animals, progressive weight gain was followed in sequence by a sustained period of increasing hyperinsulinaemia without hyperglycaemia, which was followed by overt hyperglycaemia and subsequently by declining insulin levels [7]. The results of our study show that the earliest evidence of functional and structural changes appears before the onset of hyperinsulinaemia and fasting hyperglycaemia. Development of adverse effects on the blood plasma lipid profile and glucose level appears to depend upon the duration of the HFD [53]. In fact, we observed that a high-fat intake for 24 weeks induced definite dyslipidaemia and insulin resistance as well as systemic RAS activation (unpublished data).

Although not prominent, we also observed a modest effect of HFD on renal injury in the WKY. This exacerbation of the renal inflammation without significant of BP in the WKY fed a HFD indicates that the HFD impairs renal function by pressure-independent mechanisms, as stated in a previous study [6]. As these findings also improved with Cand or tempol treatment, use of a SOD mimetic or ARB even in normotensive obese subjects might provide renoprotection.

This animal study has the following limitations. First, it is still unclear whether the oxidative stress or RAS activation comes first from the lipotoxicity in this obesity model. One study reported that the oxidative stress appears before inflammation as the primary abnormality in the kidneys of SHR [54]; the intrarenal inflammation appears to be induced primarily by the oxidative stress. Even without the use of inhibitors of the RAS, antioxidant treatment in our study eventually inhibited the intrarenal RAS activation and reduced the BP; this finding is consistent with the concept that the enhanced antagonistic action of NO, by antioxidant treatment, lowers endothelial ACE activity, generation of Ang II and endothelin-1 [46]. Second, the role of systemic RAS cannot be entirely ruled out. Even 'normal' levels of renin activity in obese hypertensive individuals might be considered as elevated, and the reason may in part be attributable to the stimulation of renin release by increased sympathetic activity [43]. However, during the early stages of obesity, intrarenal changes may precede the development of systemic RAS activation and dyslipidaemia. Further investigation on renal histological and functional changes in the later stages as well as those in the early stage of HFD-induced obesity, as reported here, is needed to elucidate the exact mechanism by which increased perirenal or intrarenal fat associated with obesity affects oxidative stress, RAS and SNS.

In conclusion, without definite systemic dyslipidaemia, insulin resistance and RAS activation, HFD induced intrarenal FFA and triglyceride accumulation and caused intrarenal RAS activation and oxidative stress, which resulted in the aggravation of hypertension and renal inflammation in the SHR. This model mimics the early presentation of obesity in humans with hypertension when fed a HFD. RAS inhibition with Cand or antioxidant treatment with tempol efficiently prevented the further elevation of BP and renal inflammation with the reduction of intrarenal lipid accumulation. Taken together, this animal study provides plausible mechanisms by which renal lipotoxicity occurs and suggests therapeutic approaches to human diseases such as obesity-related hypertension and nephropathy.

Conflict of interest statement. None declared.

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Impaired TGF-β signalling enhances peritoneal inflammation induced by *E. Coli* in rats

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Abstract

Background. Peritonitis is a common and severe complication of peritoneal dialysis (PD). Although TGF-β is a key mediator in peritoneal fibrosis with chronic PD, its role in acute peritoneal inflammation remains unclear.

Methods. Potential role of TGF-β signalling in acute peritonitis was investigated in a rat model by infecting peritoneum with *E. coli* and in primary culture of peritoneal mesothelial cells (PMC) by LPS.

Results. We found that a single infection of *E. coli* caused an acute, but transient peritonitis by a significant increase in ascites white blood cells (WBC), peritoneal CD45+ leukocytes, upregulation of TNFα, activation of NF-κB/p65 and impaired peritoneal function (all $P < 0.01$). Interestingly, spontaneous recovery of acute peritonitis occurred with upregulation of TGF-β1 and activation of Smad2/3, suggesting a protective role of TGF-β signalling in acute peritonitis. This was demonstrated by the finding that blockade of the TGF-β signalling pathway with gene transfer of Smad7 inactivated peritoneal Smad2/3 but worsened *E. coli*-induced, NF-κB-dependent peritoneal inflammation and peritoneal dysfunction (all $P < 0.01$). Furthermore, studies *in vitro* also found that impaired TGF-β signalling by overexpressing Smad7 in PMC were able to overcome the inhibitory effect of TGF-β on LPS-induced, NF-κB-mediated peritoneal inflammation.

Conclusion. Results from this study demonstrate that TGF-β signalling is essential in protection against acute peritoneal inflammation induced by bacterial infection.

Keywords: NF-κB/p65; peritonitis; Smads; TGF-β

Introduction

Recurrent peritonitis and the subsequent development of peritoneal fibrosis are the major factors associated with peritoneal failure during a long-term peritoneal dialysis (PD) [1,2]. Recurrent peritonitis, including inflammatory cell infiltration and upregulation of pro-inflammatory cytokines, often results in peritoneal fibrosis, ultimately damaging peritoneal function [1–4]. Increasing evidence shows that TGF-β is a key mediator in dialysis-related peritoneal fibrosis [3–6]. The functional importance of TGF-β in peritoneal fibrosis is well documented by the findings that overexpression of TGF-β leads to fibrosis with loss of peritoneal ultrafiltration [7,8], which is prevented by anti-TGF-β