Diabetic nephropathy and long-term treatment effects of rosiglitazone and enalapril in obese ZSF1 rats

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Abstract

Diabetic nephropathy (DN) is a major cause of end-stage renal disease. Yet the pathogenic mechanisms underlying the development of DN are not fully defined, partially due to lack of suitable models that mimic the complex pathogenesis of renal disease in diabetic patients. In this study, we describe early and late renal manifestations of DN and renal responses to long-term treatments with rosiglitazone or high-dose enalapril in ZSF1 rats, a model of metabolic syndrome, diabetes, and chronic renal disease. At 8 weeks of age, obese ZSF1 rats developed metabolic syndrome and diabetes (hyperglycemia, glucosuria, hyperlipidemia, and hypertension) and early signs of renal disease (proteinuria, glomerular collagen IV deposition, tubulointerstitial inflammation, and renal hypertrophy). By 32 weeks of age, animals developed renal histopathology consistent with DN, including mesangial expansion, glomerulosclerosis, tubulointerstitial inflammation and fibrosis, tubular dilation and atrophy, and arteriolar thickening. Rosiglitazone markedly increased body weight but reduced food intake, improved glucose control, and attenuated hyperlipidemia and liver and kidney injury. In contrast, rosiglitazone markedly increased cardiac hypertrophy via a blood pressure-independent mechanism. High-dose enalapril did not improve glucose homeostasis, but normalized blood pressure, and nearly prevented diabetic renal injury. The ZSF1 model thus detects the clinical observations seen with rosiglitazone and enalapril in terms of primary and secondary endpoints of cardiac and renal effects. This and previous reports indicate that the obese ZSF1 rat meets currently accepted criteria for progressive experimental diabetic renal disease in rodents, suggesting that this may be the best available rat model for simulation of human DN.

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Introduction

Diabetic nephropathy (DN) is a major cause of end-stage renal disease (ESRD) worldwide. The incidence of diabetes in the USA has increased by more than 50% in the past 10 years (US Renal Data System 2008) and as such constitutes an increased risk to renal health. The fact that only one-third of diabetic patients will eventually develop ESRD suggests that specific environmental, metabolic, and genetic factors must contribute to the initiation and progression of diabetic kidney disease. Despite the recent progress in our understanding of disease processes, the silent slow progression of DN has limited our success in identifying specific causative factors or factors that may predict the development of kidney disease in diabetic patients. Therefore, the complex pathogenic mechanisms involved in the development and progression of DN continue to be the subject of intense investigation. One of the barriers to full understanding of the underlying pathophysiology of DN is the lack of suitable animal models that mimic human disease (Brosius *et al*. 2009).

In the last four decades, the most commonly used model was streptozotocin (STZ)-induced type 1 diabetes in rats. Although use of this model allowed several potentially important pathogenic pathways of DN to be identified, it does not share primary features of metabolic syndrome: insulin resistance/hyperinsulinemia, obesity, and hypertension. Furthermore, STZ rats develop mild hyperlipidemia and are resistant to development of nephropathy: animals very slowly develop mild glomerular and tubulointerstitial lesions (Fioretto *et al*. 2008). Importantly, in patients with metabolic syndrome and type 2 diabetes, only one-third develop renal lesions typical of type 1 diabetes renal pathology, with diffuse and nodular

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mesangial expansion, glomerular basement membrane thickening, and with an approximately balanced severity of glomerular, tubulointerstitial, and arteriolar changes (Fioretto *et al.* 2008). Significant numbers of type 2 diabetic patients (~35%) develop an atypical pattern of renal injury with significant tubulointerstitial changes and global glomerulosclerosis (Fioretto *et al.* 2008), features not seen in STZ-induced kidney injury in rodents. Several rat models of obesity, metabolic syndrome, or type 2 diabetes that have been recently described develop more severe nephropathy, hyperlipidemia, overt proteinuria, and significant tubulointerstitial injury, and glomerulosclerosis (Michaelis *et al.* 1986, Peterson 2000, Velliquette *et al.* 2007, for review, see Tofovic & Jackson (2003)).

The obese, diabetic ZSF1 rat (Charles River, Wilmington, MA, USA) is a F1 hybrid model of type-2 DN developed by crossing rat strains with two different leptin receptor mutations (*fa* and fa^{cp}): the lean female Zucker diabetic fatty rat (ZDF; +/*fa*) and the lean male spontaneously hypertensive heart failure rat (SHHF; +/fa^{cp}) derived from the obese spontaneously hypertensive rat carrying the corpulent fa^{cp} gene (Tofovic & Jackson 2003). Both lean and obese animals inherit the gene for hypertension from the SHR strain and have similarly elevated blood pressure, but only obese ZSF1 rats (*fa*/fa^{cp}) develop dyslipidemia, hyperglycemia, and renal sclerosis and fibrosis (Tofovic *et al.* 2000, 2002, Zhang *et al.* 2007, Rafikova *et al.* 2008). Recently, Griffin *et al.* (2007) demonstrated that the development of kidney disease in the ZSF1 rat model is largely independent of hypertension and/or its potential renal transmission. Thus, the ZSF1 rat model allows for separation of renal pathophysiology strictly due to obesity, hyperglycemia, and dyslipidemia from changes due to hypertension.

Although some of the complications common in the parental strains might compromise studies of renal function and structure, i.e. hydronephrosis in ZDF rats and overt congestive heart failure in SHHF rats (McCune *et al.* 1990, Vora *et al.* 1996, Heyen *et al.* 2002, Marsh *et al.* 2007, Baynes & Murray 2009), we previously determined that obese ZSF1 rats do not develop these complications. One of the objectives of the current study was to further characterize renal disease in this model and analyze renal changes in the light of recently established criteria for DN in rodents (Brosius *et al.* 2009) and the recently published classification of DN in humans (Tervaert *et al.* 2010).

Thiazolidinediones (TZDs), including rosiglitazone, are high-affinity ligands for peroxisome proliferator-activated receptor γ (PPARγ). Long-term use of PPARγ activators improves glucose homeostasis by increasing insulin sensitivity and these agents have, until recently, been widely used in anti-diabetic therapy (Wagstaff & Goa 2002). Although PPARγ is predominantly expressed in adipocytes, it is also present in vascular and inflammatory cells, as well as renal glomerular and tubular cells (Guan & Breyer 2001), which are involved in the pathogenesis of DN. Studies on humans, particularly in diabetic patients, have suggested that PPARγ agonists significantly, although to a small extent, decrease blood pressure and proteinuria (Sarafidis *et al.* 2010). Furthermore, studies on rodent models of diabetic and non-diabetic kidney disease have shown that the renoprotective effects of TZDs may lie beyond their effects on blood pressure and glucose homeostasis (Yoshimoto *et al.* 1997, Buckingham *et al.* 1998, Ma *et al.* 2001, Tanimoto *et al.* 2004), and it has, therefore, been suggested that PPARγ agonists may show promise as therapeutic agents for diabetic renal injury.

Hypertension and increased activity of the renin–angiotensin system play central roles in the development and progression of renal damage in DN. A number of studies have suggested that use of much higher doses of angiotensin converting enzyme (ACE) inhibitors (or angiotensin II receptor blockers) than those required to reduce blood pressure may further reduce proteinuria and provide additional renal protection (Leoncini *et al.* 2010). Therefore, in this study, we used a high dose of enalapril to determine whether use of supra-maximal doses of ACE inhibitor may normalize blood pressure and prevent renal injury in diabetic ZSF1 rats.

Finally, a previous study on obese ZDF rats (a parental strain of ZSF1 rats) suggested that the PPARγ agonist rosiglitazone may provide greater renal protection than ACE inhibitors (Baylis *et al.* 2003). Therefore, in this study, we compared the relative renoprotective effects of rosiglitazone and enalapril and addressed the question of the relative contributions of blood pressure and hyperglycemia to the etiology of nephropathy in the ZSF1 rat.

Materials and Methods

A total of 40 male obese ZSF1 rats and 20 lean littermates (Charles River) were used in this study. Animals were housed at the University of Pittsburgh animal care facility (temperature 22 °C, light cycle 12 h; relative humidity 55%) and given free access to food (Purina 5008 rodent diet, Purina Mills, Land O'Lakes, St Louis, MO, USA) and water. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with institutional guidelines.

At 8 weeks of age, animals were assigned to one of the four experimental groups: lean control animals receiving no drug treatment and terminally examined for renal function at 8 or 32 weeks of age (Ln-ZSF1 group); obese control animals receiving no drug treatment and examined at 8 or 32 weeks of age (Ob-ZSF1 group); obese animals receiving enalapril in drinking water (60 mg/kg per day) and examined at 32 weeks of age (Ob-enalapril group); or obese animals receiving rosiglitazone in drinking water (5 mg/kg per day) and examined at 32 weeks of age (Ob-rosiglitazone group).

Metabolic cage studies were performed at 8, 14, 20, 26, and 32 weeks of age (i.e. at 0, 6, 12, 18, and 24 weeks of treatment) as described previously (Rafikova *et al.* 2008). Urine from the measurement period was collected and stored at −80 °C for measurement of urinary total protein, albumin, and glucose. Urinary protein and glucose were

spectrophotometrically measured with a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) and glucose hexokinase assay kit (Sigma–Aldrich) respectively. Plasma triglycerides and cholesterol (Olympus Diagnostics, Irving, TX, USA) and non-esterified fatty acids (NEFAs; Wako Chemicals USA, Inc., Richmond, VA, USA) were measured using the Olympus AU680 Clinical Chemistry Analyzer (Beckman Coulter, Brea, CA, USA). Plasma insulin was measured using an ELISA method with an antibody specific to rat insulin (Mercodia, Winston-Salem, NC, USA). Owing to the range of the ELISA, samples were diluted in deionized water. *N*-acetyl-β-(D)-glucosaminidase (NAG) activity in urine was measured using a commercial NAG assay kit (Bio-Quant, San Diego, CA, USA). Urinary albumin concentrations were measured by Rat Microalbumin Assay (Kamiya Biomedical Company, Seattle, WA, USA).

At baseline and at 32 weeks of age, animals were instrumented for measurement of renal hemodynamic and excretory function as described previously (Tofovic *et al.* 2002). Briefly, rats were anesthetized with pentobarbital (45 mg/kg i.p.) and a PE-240 catheter was inserted in the trachea to facilitate breathing. A PE-50 catheter connected to a blood pressure analyzer (Micro-Med, Inc., Louisville, KY, USA) was inserted in the right carotid artery for measurement of mean arterial blood pressure (MABP) and heart rate (HR). Two PE-50 catheters were inserted in the right jugular vein for administration of saline (50 μl/min) and supplemental anesthetic. An abdominal midline incision was made and a flow probe (Model 1RB; Transonic Systems, Inc., Ithaca, NY, USA) was placed on the left renal artery for measurement of renal blood flow (RBF). The left ureter was cannulated with PE-10 tubing to facilitate collection of urine. I.v. infusion of ^{14}C-inulin (0·035 μCi/20 μl per min) was initiated, and a 1 h stabilization period was permitted before two 30 min clearance periods were conducted. During each clearance period, urine excreted from the left kidney was collected; a 200 μl midpoint blood sample was taken for measurement of hematocrit and radioactivity; MABP, HR, and RBF were recorded at 1 min intervals and averaged, and renal vascular resistance (RVR) and renal plasma flow (RPF) were calculated. Urine volume (UV) for each period was gravimetrically determined and plasma and urine ^{14}C radioactivity were measured with a liquid scintillation analyzer (model 2500TR; Packard Instrument Company, Downers Grove, IL, USA). Renal clearance of ^{14}C-inulin was calculated as an estimate of glomerular filtration rate (GFR). At the conclusion of the second clearance period, a terminal blood sample was taken and the animal was killed with an anesthetic overdose. The right kidney, heart, liver, and brain were excised, washed in ice-cold PBS, and weighed. The right kidney was bisected, and one half was flash frozen in liquid nitrogen and the other fixed in 10% buffered formalin for histopathological and immunohistochemical studies. Samples of heart (longitudinal section of left ventricle) and liver (left lateral lobe) were fixed in 10% buffered formalin.

Histopathology

The right kidney tissue sample stored in 10% formalin was sectioned and then processed into paraffin blocks and analyzed by light microscopy and immunohistochemistry. The kidney was cut into two histological sections (5 μm thick) and stained with hematoxylin–eosin (H&E) and methenamine silver-trichrome respectively. Kidney slices were examined by light microscopy and scored in a blinded fashion by one of the investigators (S B). A total of at least 350 glomeruli from each rat were studied and the percentage of glomeruli showing focal segmental glomerulosclerosis (FSGS part of the tuft) and focal global glomerulosclerosis (FGGS whole tuft) was determined.

Renal cortical segments (5 μm) were incubated overnight at 4 °C with rabbit anti-mouse collagen IV antibody (dilution 1/500) obtained from Chemicon International, Inc. (Temecula, CA, USA). A rat ED1 antibody (Serotec, Raleigh, NC, USA) specific for a monocyte/macrophage cytoplasmic antigen was used to label glomerular and interstitial macrophages. Nonspecific staining was assessed by replacing the primary antibody with PBS. Sections were washed and further developed according to the directions of the manufacturer (Dako Corporation, Carpinteria, CA, USA) using the LSAB2 Kit that contained a second antibody linked to avidin- and peroxidase-conjugated biotin. Immunohistochemical staining for collagen IV was assessed quantitatively with a SAMBA 4000 image analyzer (Image Products International, Chantilly, VA, USA), using specialized software (Immuno-Analysis, version 4.1, Microsoft), a color video camera, and a Compaq computer. The software designed for immunostaining analysis

Table 1 Baseline metabolic and renal function parameters in 8-week-old lean and obese ZSF1 rats (*$P<0·05$)

Parameters	**Lean** ($n=20$)	**Obese** ($n=20$)
Body weight (g)	269±5	332±10*
Food intake (g/kg per day)	82·3±2·1	125·6±2·2*
Fluid intake (ml/kg per day)	131·2±6·8	275·2±18·8*
Urine volume (ml/kg per day)	36·0±3·0	164·8±14·5*
Blood glucose (mg/dl)	114·4±2·8	147·2±3·9*
Plasma cholesterol (mg/dl)	50±2	74±4*
Plasma triglycerides (mg/dl)	49±3	483±46*
Plasma insulin (nM)	0·32±0·05	3·18±0·68*
Glycosylated hemoglobin (%)	4·7±0·03	6·0±0·1*
Urine glucose (g/kg per day)	0·22±0·002	7·48±1·28*
Urinary creatinine excretion (mg/kg per day)	40·2±2·3	49·3±1·6*
Urinary protein excretion (mg/kg per day)	92·6±6·4	307±23*
Urine protein/creatinine ratio	2·48±0·09	5·98±0·41*
Urinary albumin excretion (mg/kg per day)	1·24±0·19	11·27±1·91*
Urine albumin/creatinine ratio	0·03±0·001	0·23±0·04*
Urinary NAG excretion (mU/kg per day)	409±30	866±61*
Urine NAG/creatinine ratio	10·2±0·3	17·4±1·0*

enabled the operator to set density threshold values by averaging several fields on the negative control tissues in which the primary antibody was replaced with PBS. Background subtraction was then automatically performed on every tissue. Cryostat sections were stained with oil red O to evaluate the renal accumulation of neutral lipids. The results are reported as the percentage of the total examined area that stained positive. Ten high-power fields (400×) were examined for staining density or positively marked cells for ED-1.

Heart and liver samples taken at necropsy and fixed in 10% formalin were conventionally processed in paraffin wax. Tissue sections (4–5 μm thick) were stained with H&E and assessed by light microscopy.

Statistical analysis

All data are presented as mean±S.E.M. Statistical analyses were performed using the Number Cruncher Statistical Software program (Kaysville, UT, USA). Group comparisons for data from metabolic studies (repeated measurements) were performed using a one (1F)- or two (2F)-factor hierarchical ANOVA as appropriate, followed by a Fisher's least significant difference (LSD) test for *post hoc* comparisons. Comparison of data from acute experiments and from histological analysis (single point data) was performed by 1F-ANOVA (four groups at 32 weeks of age) or by Student's *t*-test (baseline measurements in lean versus obese animals at 8 weeks of age). Probability value $<0{\cdot}05$ was considered statistically significant. All data from semiquantitative histopathological analysis were analyzed by the non-parametric Mann–Whitney *U* test.

Table 2 Renal hemodynamics and excretory function in 8-week-old lean and obese ZSF1 rats (*$P<0{\cdot}05$)

Parameters	**Lean** ($n=10$)	**Obese** ($n=10$)
Body weight (g)	287±11	361±15*
Kidney weight (g)	1·10±0·03	1·41±0·13*
Kidney/body weight ratio (g/kg)	3·87±0·12	3·94±0·38
Mean blood pressure (mmHg)	140·7±3·1	141·7±2·9
Renal blood flow (ml/min)	7·72±0·9	6·54±0·78
Renal plasma flow (ml/min)	4·16±0·50	3·70±0·44
Renal vascular resistance (mmHg/ml per min)	20·6±2·3	24·3±2·3
Urine volume (μl/min)	9·2±1·6	13·8±1·0*
Glomerular filtration rate (ml/min)	1·75±0·13	1·73±0·17
Filtration fraction	0·480±0·063	0·545±0·080
Renal blood flow (ml/min per g kidney)	7·06±0·87	5·01±0·72
Renal plasma flow (ml/min per g kidney)	3·81±0·48	2·82±0·40
Renal vascular resistance (mmHg/ml per min per g kidney)	22·5±2·39	34·8±5·1
Urine volume (μl/min per g kidney)	8·79±1·73	10·4±1·57
Glomerular filtration rate (ml/min per g kidney)	1·61±0·13	1·29±0·15

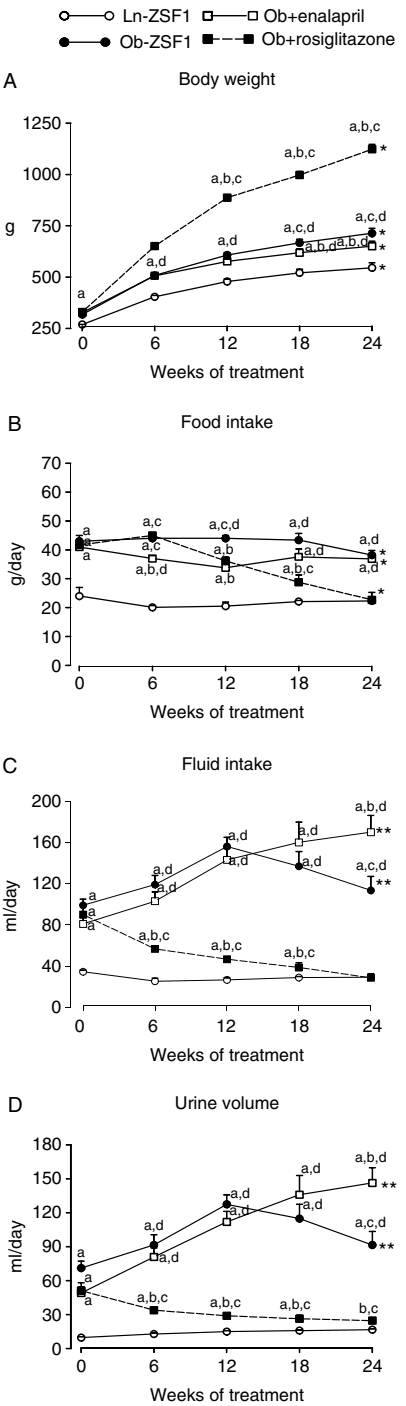

Figure 1 The effects of rosiglitazone and enalapril on body weight (A), food (B) and fluid (C) intake, and urine volume (D) in obese, diabetic ZSF1 rats (2F-ANOVA: (A) Treatment, $P<0{\cdot}001$, (B) Time, $P<0{\cdot}001$, A×B interaction, $P<0{\cdot}001$. Fisher's LSD test: treatment effects, $P<0{\cdot}05$, *, versus all other groups; **, versus lean and Ob-rosiglitazone groups; time point treatment effects, $P<0{\cdot}05$, a, versus lean; b, versus Ob; c, versus Ob-enalapril; and d, versus Ob-rosiglitazone group respectively).

Results

Baseline metabolic, cardiovascular, and renal parameters in young (8-week-old) obese and lean ZSF1 rats

Baseline measurements of metabolic parameters and renal hemodynamics and excretory function in lean and obese ZSF1 rats are presented in Tables 1 and 2 and Figs 1, 2, 3 and 4. At 8 weeks of age, body weights, and food consumption were already significantly higher in obese ZSF1 rats than in lean littermates (Table 1) as described previously (Rafikova *et al.* 2008). Obese ZSF1 rats were diabetic at this time. Although obese animals had only moderately increased fasted blood glucose and glycosylated hemoglobin (HbA1c) levels, they showed marked polydipsia, polyuria, and glucosuria, were hyperinsulinemic, and had elevated plasma triglycerides, cholesterol, and NEFA levels (Figs 3 and 4).

Consistent with our previous studies (Tofovic & Jackson 2003, Rafikova *et al.* 2008), both lean and obese animals were hypertensive and had similarly elevated arterial blood pressure and similar RBF, RPF, RVR, and GFR (Table 2). Obese ZSF1 rats already exhibited renal hypertrophy and tended ($P<0{\cdot}06$) to have increased renal vascular resistance when RVR was corrected for kidney weight (Table 2, lower part). At 8 weeks of age, both lean and obese animals had normal renal histopathology except for high (90–100%) incidence of minimal/mild tubular dilatation and low incidence (10%) of moderate hydronephrosis. The onset of diabetes was associated with early renal injury. Obese rats had increased urinary protein, urinary albumin excretion, and urine NAG content (Table 1). Furthermore, mild but statistically significant increase in glomerular expression of collagen IV (Fig. 6C) and tubulointerstitial inflammation (ED1+ cells; Fig. 6E) were detected in obese animals.

Effects of rosiglitazone and enalapril on body weight and metabolic status

The effects of 24-week treatment with rosiglitazone or enalapril on metabolic parameters in obese ZSF1 rats are presented in Figs 1, 2 and 3. At baseline (8 weeks of age) and at 6 weeks of the experiment, food consumption was similar in all obese animals, but Ob-rosiglitazone group gained more weight than Ob-ZSF1 group or Ob-enalapril group (Fig 1A and B). After 12 weeks of dosing, Ob-rosiglitazone rats had significantly reduced food intake, and by the end of the study, food intake in Ob-rosiglitazone group was similar to daily food intake in lean rats (Ln-ZSF1 group). Notably, the Ob-rosiglitazone group continued to gain more weight than obese controls during the study. After 24 weeks of treatment, mean group body weights were as follows: Ob-ZSF1 group, 657 ± 15 g ($P<0{\cdot}001$ versus Ln-ZSF1); Ob-rosiglitazone group, 1122 ± 13 g ($P<0{\cdot}001$ versus Ob-ZSF1); Ob-enalapril group, 604 ± 16 g ($P<0{\cdot}05$ versus Ob-ZSF1); and Ln-ZSF1, 530 ± 7 g. At 32 weeks of age, Ob-rosiglitazone animals were severely obese and showed 64·7% increase in body weight compared with the Ob-ZSF1 group.

At 8 weeks of age, Ob-ZSF1 rats exhibited moderately increased blood glucose levels compared with lean controls

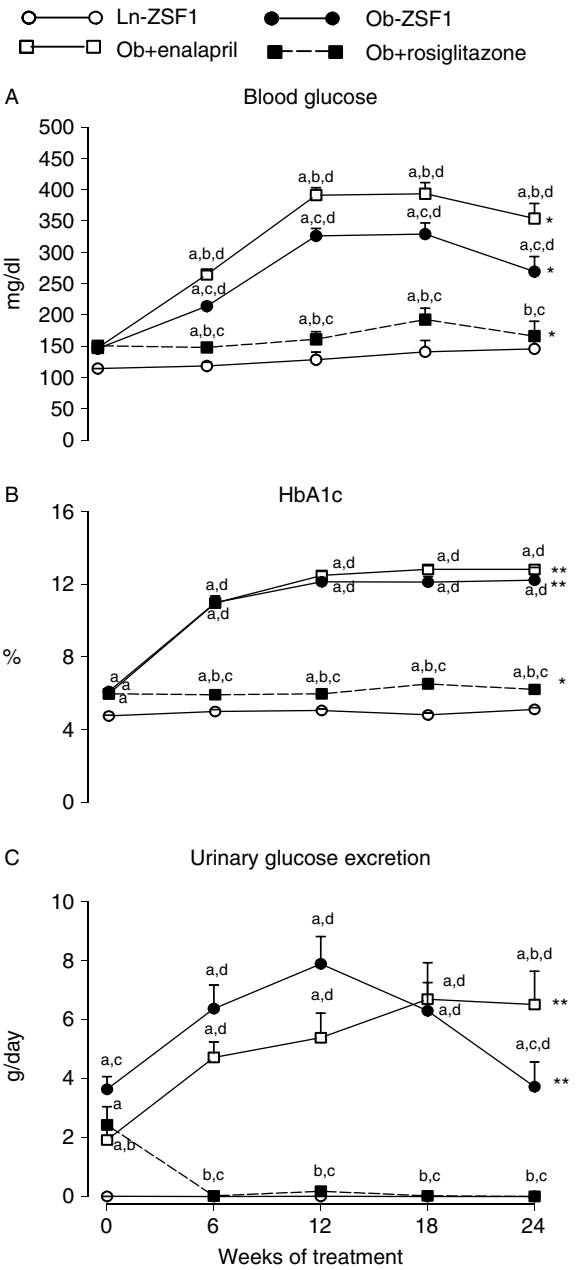

Figure 2 The effects of rosiglitazone and enalapril on blood glucose (A), HbA1c (B) and glucosuria (C) in obese, diabetic ZSF1 rats (2F-ANOVA: (A) Treatment, $P<0{\cdot}001$, (B) Time, $P<0{\cdot}001$, A×B interaction, $P<0{\cdot}001$. Fisher's LSD test: treatment effects, $P<0{\cdot}05$, *, versus all other groups; **, versus lean and Ob-rosiglitazone groups; time point treatment effects, $P<0{\cdot}05$, a, versus lean; b, versus Ob; c, versus Ob-enalapril; and d, versus Ob-rosiglitazone group respectively).

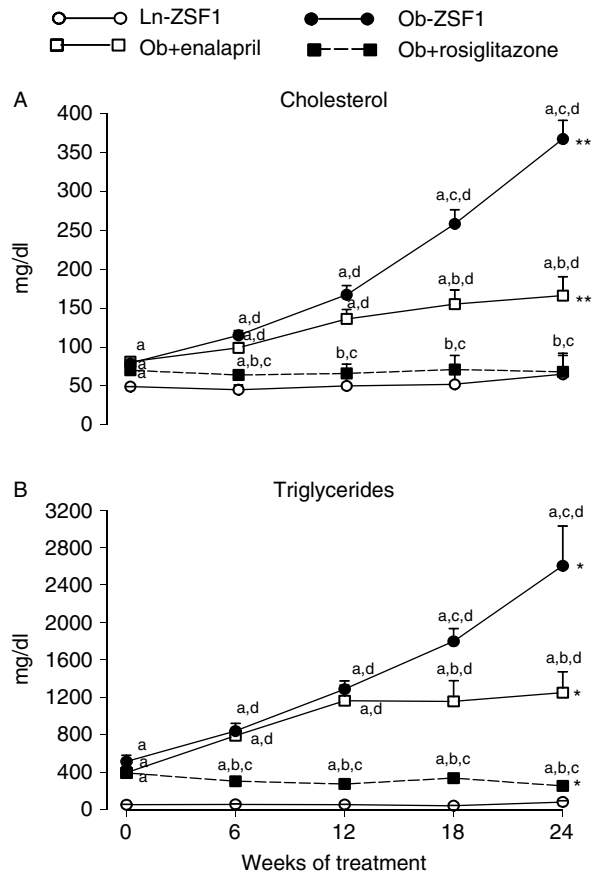

Figure 3 The effects of rosiglitazone and enalapril on plasma cholesterol (A) and triglycerides (B) in obese, diabetic ZSF1 rats (2F-Anova: (A) Treatment, $P<0{\cdot}001$, (B) Time, $P<0{\cdot}001$, A×B interaction, $P<0{\cdot}001$. Fisher's LSD test: treatment effects, $P<0{\cdot}05$, *, versus all other groups; **, versus lean and Ob-rosiglitazone groups; time point treatment effects, $P<0{\cdot}05$, a, versus lean; b, versus Ob; c, versus Ob-enalapril; and d, versus Ob-rosiglitazone group respectively).

(114±4 vs 147±4 mg/dl; Ln-ZSF1 versus Ob-ZSF1). Rosiglitazone treatment prevented further worsening of hyperglycemia, and after 24 weeks, rosiglitazone-treated animals and lean controls had comparable, although statistically significantly different, blood glucose levels (166±4 vs 137±5 mg/dl; Ob-rosiglitazone versus Ln-ZSF1; $P<0{\cdot}05$) and HbA1c levels (6·2±0·2 vs 5·1 ±0·1%; Ob-rosiglitazone versus Ln-ZSF1, $P<0{\cdot}05$).

Both Ob-ZSF1 and Ob-enalapril groups had markedly elevated blood glucose and HbA1c levels (blood glucose: 270±17 and 354±4 mg/dl; HbA1c: 12·2±0·3 and 12·8 ±0·1%; Ob-ZSF1 and Ob-enalapril groups respectively). Enalapril-treated animals had increased blood glucose levels compared with the Ob-ZSF1 group. Although this may be somewhat surprising, it has been previously reported that enalapril may increase blood glucose levels in both the obese Zucker rat (Oltman *et al.* 2008*a*) and the ZSF1's parental strain, the obese ZDF rat (Oltman *et al.* 2008*b*).

Improved glucose control in Ob-rosiglitazone rats was associated with markedly reduced polydipsia and polyuria and disappearance of glucosuria (Fig. 2C), whereas enalapril had no effect on these parameters of glucose control.

The elevated plasma lipids in 8-week-old obese rats continued to increase over the next 24 weeks and, at 32 weeks of age, the obese rats showed a sixfold increase in plasma cholesterol and triglyceride levels compared with levels at 8 weeks of age. Remarkably, treatment with rosiglitazone corrected hyperlipidemia, i.e. prevented time-related increases in lipid levels: Ob-rosiglitazone and Ln-ZSF1 groups had similar plasma total cholesterol, triglycerides, and NEFA levels (Figs 3A and B and 4B respectively). Old obese rats were much less hyperinsulinemic than young obese animals (3·18±0·68 vs 0·99±0·12 nM, 8-week-old versus 32-week-old obese rats), suggesting incipient β-cell insufficiency. Treatment with enalapril had delayed effects on plasma lipids, namely, reduced total plasma cholesterol and triglyceride levels after 12 and 24 weeks of treatment (Fig 3A and B), and had no effect on NEFA plasma level (Fig. 4B).

Effects of rosiglitazone and enalapril on blood pressure and renal function

Before initiating treatments, at 8 weeks of age, both obese and lean control animals were hypertensive (Table 1). During the next 24 weeks, blood pressure increased slightly, and at 32 weeks of age, MABP was 157±2 and 154±3 mmHg in lean and obese rats respectively (Table 3). Obese rats treated with rosiglitazone for 24 weeks had significantly lower mean blood pressure (136±6 mmHg) compared with lean and obese controls. Notably, high-dose enalapril reduced MABP by ∼50 mmHg to normotensive levels (Ob-enalapril group, MABP=108±2 mmHg).

Obese animals developed progressive renal disease and, by 32 weeks of age, Ob-ZSF1 rats had elevated albuminuria (25-fold) and urinary NAG activity (three-fold), a marker of tubular damage, compared to lean littermates (Fig. 5). High-dose enalapril nearly eliminated

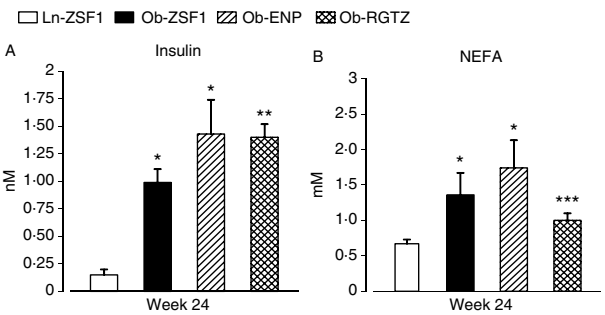

Figure 4 The effects of rosiglitazone and enalapril on plasma insulin (A) and non-esterified fatty acid (NEFA) lipids (B) in obese, diabetic ZSF1 rats. 1F-ANOVA: $P<0{\cdot}001$. *, versus Ln-ZSF1; **, versus Ln- & Ob-ZSF1; ***, versus all other groups.

Table 3 Renal hemodynamics and excretory function in 32-week-old lean (Ln-ZSF1) and obese (Ob-ZSF1) ZSF1 rats and in obese animals treated with enalapril or rosiglitazone for 24 weeks (Ob-ENP, Ob-RGTZ; Fisher *post hoc* LSD test: a, versus all other groups; b, versus Ln-ZSF1 and Ob-ZSF1; c, versus Ln-ZSF1; d, versus Ln-ZSF1 and Ob-ENP; e, versus Ob-ZSF1; f, versus Ln-ZSF1 and Ob-RGTZ)

Parameters	**Ln-ZSF1** ($n=10$)	**Ob-ZSF1** ($n=10$)	**Ob-ENP** ($n=10$)	**Ob-RGTZ** ($n=10$)	**1F-ANOVA** ($P<$)
Body weight (g)	542±8	677±21[a]	618±17[a]	1141±15[a]	0·001
Kidney (g)	1·73±0·43	3·06±0·15[a]	2·13±0·06[a]	2·47±0·09[a]	0·001
MABP (mmHg)	157·4±2·3	154·0±3·0	108·2±1·7[a]	136·0±5·9[a]	0·001
RBF (ml/min)	7·70±0·33	9·06±0·51	10·8±0·46[b]	10·8±0·50[b]	0·001
RPF (ml/min)	4·04±0·16	5·33±0·33[c]	6·40±0·34[b]	6·42±035[b]	0·001
RVR (mmHg/ml per min)	20·9±1·0	17·7±1·2	10·2±0·4[b]	12·9±0·9[b]	0·001
UV (µl/min)	8·2±1·5	21·7±4·7[a]	36·1±6·3[a]	6·6±0·8	0·001
GFR (ml/min)	2·24±0·15	2·61±0·15	2·69±0·15	2·24±0·22	0·184
Filtration fraction	0·57±0·05	0·49±0·04	0·41±0·03[c]	0·36±0·04[b]	0·011
RBF (ml/g kidney per min)	4·47±0·21	3·03±0·22[a]	5·42±0·35[a]	4·46±0·29	0·001
RPF (ml/g kidney per min)	2·35±0·11	1·78±0·14[a]	3·10±0·18[b]	2·64±0·17[e]	0·001
RVR (mmHg/ml per g kidney per min)	35·8±1·52	54·3±4·73[a]	21·0±1·15[a]	32·0±2·70	0·001
UV (µl/g kidney per min)	4·87±0·93	6·77±1·20	19·4±3·67[a]	2·70±0·38	0·001
GFR (ml/g kidney per min)	1·31±0·10	0·87±0·07[d]	1·28±0·08	0·93±0·11[d]	0·004
RBF (ml/g brain per min)	3·73±0·17[a]	4·62±0·27[a]	5·85±0·23[b]	5·74±0·27[b]	0·001
RPF (ml/g brain per min)	1·96±0·08[a]	2·72±0·18[a]	3·47±0·18[b]	3·40±0·16[b]	0·001
RVR (mmHg/ml per g brain per min)	42·9±1·77[a]	34·7±2·44[a]	18·8±0·79[b]	24·3±1·70[b]	0·001
UV (µl/g brain per min)	4·01±0·74	11·3±2·52[f]	19·8±3·56[f]	3·51±0·45	0·001
GFR (ml/g brain per min)	1·09±0·08	1·34±0·08[f]	1·47±0·09[f]	1·19±0·13	0·001

albuminuria and reduced NAG activity, whereas rosiglitazone reduced albuminuria by 90% and had no effect on NAG activity. Both Ob-rosiglitazone and Ob-enalapril rats showed increased RBF and decreased RVR compared with obese controls (Table 3). The effects of enalapril were profound: normalization of blood pressure in obese animals was associated with ∼20% increase in RBF and ∼45% reduction in RVR compared with obese rats. The effects of enalapril were even more remarkable when parameters were normalized by kidney weight, and normalization of blood pressure by enalapril was associated with increased RBF by +79% and +47·5% compared with Ob-ZSF1 and Ln-ZSF1 groups respectively. Similarly, in the Ob-enalapril group, RVR was reduced by 61·3 and 41·7% compared with Ob-ZSF1 and Ln-ZSF1 groups respectively. The Ln-ZSF1 and Ob-rosiglitazone groups had similar GFR, whereas Ob-ZSF1 and Ob-enalapril groups tended to have higher GFR.

Both rosiglitazone and enalapril reduced urinary protein excretion and almost prevented albuminuria. Enalapril, but not rosiglitazone, reduced NAG activity in urine (Fig. 5).

The effects of rosiglitazone and enalapril on renal histopathology

Data analyses for renal morphology, histochemistry, and immunohistochemistry in 32-week-old Ln-ZSF1 and Ob-ZSF1 rats are presented in Fig. 6 and Tables 4, 5 and 6. Normal histology in Ln-ZSF1 rats, major pathological features of severe nephropathy in Ob-ZSF1 rats, and the effects of rosiglitazone and enalapril on renal structure in diabetic kidneys are given (Figs 7 and 8).

At 32 weeks of age, lean animals had normal renal histology, with the exception of mild cortical tubular dilatation that was also seen in young lean and obese animals. In contrast, the majority (9/10) of adult Ob-ZSF1 controls showed substantial cortical tubular dilatation with intratubular protein casts. Hydronephrosis was not detected in lean animals, and severe hydronephrosis was present in only one Ob-ZSF1 rat. Renal histological assessment of 32-week-old animals revealed a significant degree of chronic renal disease in obese, hypertensive, and diabetic rats compared to their age-matched lean, hypertensive, and non-diabetic littermates. The tubulointerstitial changes in Ob-ZSF1 rats involved moderate interstitial fibrosis and inflammation and markedly dilated tubules containing eosinophilic proteinaceous casts, which also exhibited either flattened or atrophic tubular epithelium and, in some cases, protein resorption droplets in proximal tubular epithelial cells (Fig. 8G). In obese diabetic rats, glomeruli often showed evidence of segmental or global glomerulosclerosis (Figs 6D and 8E); the incidence of glomerulosclerosis was 6·5% in the Ob-ZSF1 group.

The quantitative analysis of kidney sections stained with oil red O revealed significant deposition of neutral lipids in glomeruli and tubulointerstitium of Ob-ZSF1 rats, compared to the minimal presence of neutral lipids in glomeruli and tubulointerstitium of lean littermates (Fig. 6A and B and 7 bottom photomicrographs). Consistent with increased glomerulosclerosis, obese rats showed increased glomerular expression of collagen IV, whereas lean rats had mildly increased glomerular collagen IV content (Fig. 6C and 7 top photomicrographs). Finally, significant inflammation, i.e. influx of macrophages, was detected in glomeruli and tubulointerstitium in kidneys from obese rats (Figs 6E and F and 7).

In all Ob-enalapril animals, nephropathy was present but at lesser severity than in the Ob-ZSF1 controls, as illustrated by

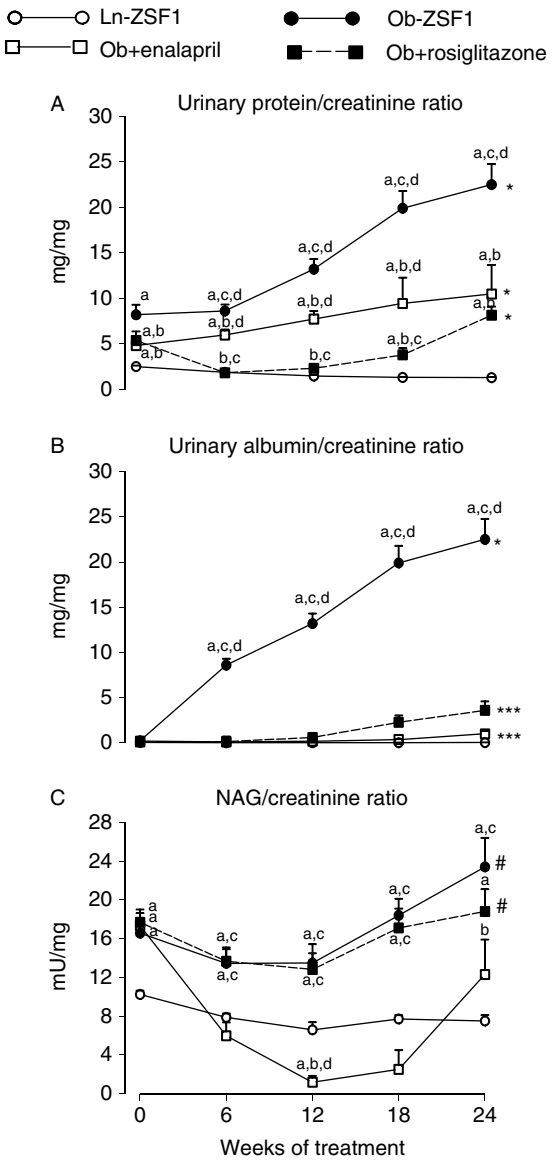

Figure 5 The effects of rosiglitazone and enalapril on urinary protein (A) and albumin (B) excretion and *N*-acetyl-β-D-glucosaminidase (NAG) activity (C) in obese diabetic ZSF1 rats (2F-ANOVA: (A) Treatment, $P<0{\cdot}001$, (B) Time, $P< 0{\cdot}001$, A×B interaction, $P<0{\cdot}001$. Fisher's LSD test: treatment effects, $P<0{\cdot}05$, *, versus all other groups; **, versus lean and Ob-rosiglitazone groups; ***, versus Ob-ZSF1; #, versus Ln-ZSF1 and Ob-enalapril groups; time point treatment effects, $P<0{\cdot}05$, a, versus lean; b, versus Ob; c, versus Ob-enalapril; and d, versus Ob-rosiglitazone group respectively).

the less severe cortical dilatation present. Hydronephrosis of minimal severity and pyelonephritis graded from minimal to moderate were seen in 1 and 3 animals of a total of 9 respectively (Table 6). The Ob-enalapril group alone showed cortical arterial medial hypertrophy/sclerosis of generally mild severity (Fig. 8I).

In the Ob-rosiglitazone group, nephropathy was seen in all animals at lesser severity than the Ob-ZSF1 controls with no cortical arterial medial hypertrophy/sclerosis observed. Hydronephrosis at mild severity was seen in a minority of Ob-rosiglitazone animals (2/10) and moderately severe pyelonephritis was seen in one Ob-rosiglitazone animal.

Rosiglitazone prevented, and enalapril significantly reduced, renal lipid accumulation (Figs 6A and B and 7, lower photomicrographs). Both treatments inhibited glomerular expression of collagen IV (Figs 6C and 7 upper photomicrographs) and markedly reduced the incidence of glomerulosclerosis (Figs 6D and 8E and I). Finally, rosiglitazone and enalapril almost eliminated glomerular and tubulointerstitial inflammation, i.e. the influx of ED1+ cells (Figs 6E and F and 7 center photomicrographs).

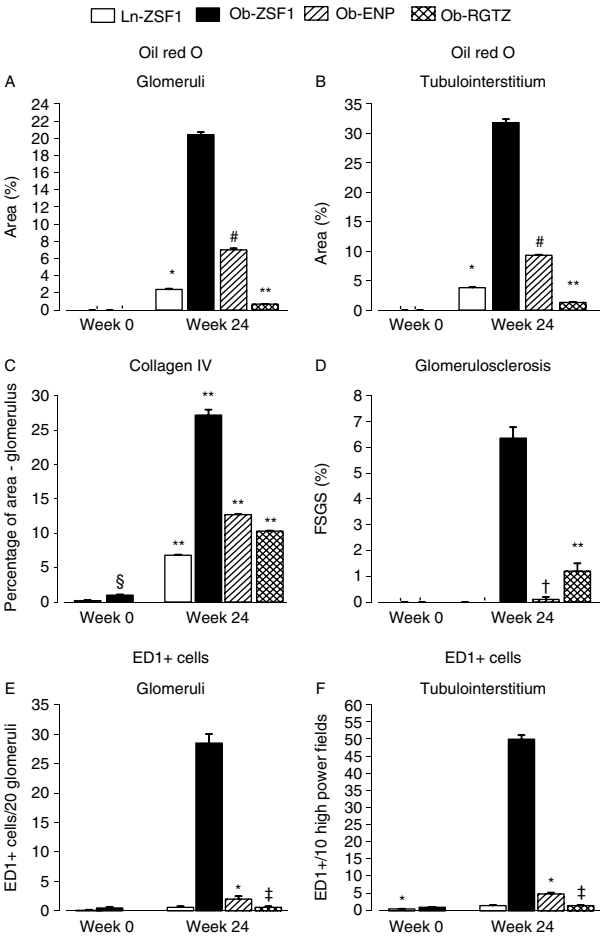

Figure 6 Renal deposition of neutral lipids (A, B) and fibrotic (C, D) and inflammatory changes (E, F) in 32-week-old lean and obese ZSF1 rats and obese animals treated with rosiglitazone or enalapril for 24 weeks. 1F-ANOVA: $P<0{\cdot}001$. *, versus Ob; **, versus all other groups; #, versus Ob & Ln; §, versus Ob Wk-0; †, versus Ob & RGTZ; ‡, versus Ob & ENP.

Table 4 Glomerular and tubulointerstitial injury in 32-week-old lean (Ln-ZSF1) and obese (Ob-ZSF1) ZSF1 rats and in obese animals treated with enalapril or rosiglitazone for 24 weeks (Ob-enalapril, Ob-rosiglitazone)

	n	Number of examined glomeruli	Glomerulosclerosis			Protein casts (0–3+)	Tubular atrophy (0–3+)	Interstitial inflammation (0–3+)	Interstitial fibrosis (0–3+)	Arterial sclerosis (0–3+)	Arteriolar sclerosis (0–3+)
			Segmental (%)	Global (%)	Total (%)						
Ln-ZSF1	10	447	0	0	0	0·1	0	0	0	0·3	0·4
		23				0·1			0	0·1	0·1
Ob-ZSF1	10	403	5·54±0·41	0·81±0·15	6·36±0·42	1·9	1·3±0·1	1·10±0·10	0·4	0·4±0·1	0·70±0·2
		16				0·1			0·1		
Ob-enalapril	9	390	0	0	0	0·3±0·1	0·0±0·0	0·10±0·03	0·00	0·2±0·1	2·30±0·1
		29									
Ob-rosiglitazone	10	427	1·1±0·3	0·1±0·1	1·2±0·3	0·5±0·1	0·4±0·1	0·2±0·1	0·1±0·1	0·1±0·1	0·3±0·1
		30									
1F-ANOVA	*P*<	0·612	0·001	0·001	0·001						
Non-parametric Mann–Whitney *U* test						0·05	0·05	0·05	0·05	0·315	0·05

Heart and liver histopathology

Heart and liver morphology and histopathology data are presented in Tables 5 and 6 and Fig. 9. At 32 weeks of age, Ob-ZSF1 rats had only moderately increased heart weight compared to lean littermates, with significant heart weight increase only when heart weight was normalized by brain weight.

Myocardial microvesicular steatosis was present at minimal/mild severity in ~50% of Ob-ZSF1 and Ob-enalapril rats. In lean animals, this feature was absent and observed at minimal severity in one Ob-rosiglitazone animal only (Fig. 9E–H). Myocarditis was present in the majority of Ob-ZSF1 rats and Ob-enalapril animals at minimal/mild severity, but also, in a minority and at minimal severity in Ln-ZSF1 controls. Myocarditis was present in all Ob-rosiglitazone animals and at mild severity in the majority (9/10).

All Ob-ZSF1 and Ob-enalapril animals at 32 weeks of age were characterized by mild to severe diffuse hepatocyte fat vacuolation (i.e. in all hepatocytes from periportal to centrilobular regions within the tissue section; Fig. 9A) with associated glycogen vacuolation. Ln-ZSF1 rats exhibited neither hepatocyte fat nor glycogen vacuolation (Fig. 9B). By contrast, the majority (7/10) of Ob-rosiglitazone animals showed minimal diffuse hepatocyte vacuolation with the remaining exhibiting mild diffuse hepatocyte fat vacuolation in centrilobular hepatocytes only (Fig. 9D). A majority of Ob-rosiglitazone animals (7/10) exhibited an absence of hepatocyte glycogen vacuolation. In a minority (3/10) of Ob-rosiglitazone animals, hepatocyte eosinophilic hyaline droplets were observed. Foci of hepatocellular necrosis with inflammatory cell infiltrates at minimal/mild severity were seen in a minority of these animals.

Discussion

One important finding of this study is that obese ZSF1 rats develop metabolic syndrome and diabetes (hyperglycemia, glucosuria, hyperlipidemia, and hypertension) as early as 8 weeks of age and that metabolic changes are associated with early signs of renal disease: proteinuria, glomerular deposition of collagen IV, tubulointerstitial inflammation, and renal hypertrophy. Consistent with our previous findings (Tofovic & Jackson 2003, Rafikova *et al.* 2008), both obese and lean animals developed hypertension by 8 weeks of age and had severe hypertension by 32 weeks of age. The presence of hypertension in both lean and obese animals is not surprising, as both strains inherit genes for spontaneous hypertension from a parental strain, the SHHF/Mcc-*fa*^{cp} (McCune *et al.* 1994, Tofovic & Jackson 1999). Another important observation is that, in contrast to obese ZDF rats (the other parental strain), both lean and obese ZSF1 rats in this study had a very low incidence (10%) of hydronephrosis. Also, regardless of phenotype, all animals in this study showed minimal to moderate cortical tubular dilatation. Because these renal changes occurred at an early age and were present in both lean

Table 5 Organ weight in 32-week-old lean (Ln-ZSF1) and obese (Ob-ZSF1) ZSF1 rats and in obese animals treated with enalapril or rosiglitazone for 24 weeks (Ob-enalapril, Ob-rosiglitazone). Fisher's LSD test: $P<0{\cdot}05$; a, versus all other groups; b, versus Ob-ZSF1 and Ob-rosiglitazone; c, versus Ob-rosiglitazone; d, versus Ln-ZSF1 and Ob-rosiglitazone; BW, body weight

Group	*n*	Heart (g)	Liver (g)	Kidney (g)	Heart/BW (mg/g)	Heart/Brain (mg/g)	Liver/BW (mg/g)	Liver/Brain (mg/g)	Kidney/BW (mg/g)	Kidney/Brain (mg/g)
Ln-ZSF1	10	$1{\cdot}53\pm0{\cdot}04^{c}$	$13{\cdot}74\pm0{\cdot}44^{a}$	$1{\cdot}73\pm0{\cdot}04^{a}$	$2{\cdot}83\pm0{\cdot}07^{a}$	$0{\cdot}74\pm0{\cdot}01^{b}$	$25{\cdot}3\pm0{\cdot}54$	$6{\cdot}64\pm0{\cdot}18^{a}$	$3{\cdot}19\pm0{\cdot}06^{b}$	$0{\cdot}84\pm0{\cdot}01^{a}$
Ob-ZSF1	10	$1{\cdot}69\pm0{\cdot}03$	$40{\cdot}76\pm1{\cdot}42^{a}$	$3{\cdot}06\pm0{\cdot}13^{a}$	$2{\cdot}52\pm0{\cdot}06$	$0{\cdot}86\pm0{\cdot}01^{d}$	$60{\cdot}5\pm2{\cdot}10^{a}$	$20{\cdot}83\pm0{\cdot}88^{a}$	$4{\cdot}56\pm0{\cdot}23^{a}$	$1{\cdot}56\pm0{\cdot}08^{a}$
Ob-enalapril	9	$1{\cdot}46\pm0{\cdot}10^{b}$	$32{\cdot}87\pm2{\cdot}24^{a}$	$2{\cdot}13\pm0{\cdot}06^{a}$	$2{\cdot}35\pm0{\cdot}13$	$0{\cdot}79\pm0{\cdot}05^{c}$	$52{\cdot}8\pm2{\cdot}4^{a}$	$17{\cdot}72\pm0{\cdot}10^{a}$	$3{\cdot}45\pm0{\cdot}08^{b}$	$1{\cdot}15\pm0{\cdot}02^{a}$
Ob-rosiglitazone	10	$2{\cdot}29\pm0{\cdot}10^{a}$	$28{\cdot}19\pm1{\cdot}03^{a}$	$2{\cdot}47\pm0{\cdot}08^{a}$	$2{\cdot}00\pm0{\cdot}07^{a}$	$1{\cdot}21\pm0{\cdot}05^{a}$	$24{\cdot}7\pm1{\cdot}0$	$14{\cdot}90\pm0{\cdot}51^{a}$	$2{\cdot}17\pm0{\cdot}07^{a}$	$1{\cdot}30\pm0{\cdot}04^{a}$
1F-ANOVA	$P<$	0·001	0·001	0·001	0·001	0·001	0·001	0·001	0·001	0·001

and obese animals, they could not be related to metabolic syndrome and are most likely inherited from the maternal ZDF strain. In this regard, high incidence (40–50%) of moderate to severe hydronephrosis has been reported in both lean and obese ZDF rats (Vora *et al.* 1996, Marsh *et al.* 2007). Detection of minimal (but statistically significant) expression of collagen IV in glomeruli and ED1+ cells in the tubulointerstitium in young obese ZSF1 rats suggests incipient renal injury due to metabolic syndrome, and this phenotype of obese ZSF1 rats, present at 8 weeks of age, is consistent with the metabolic syndrome and DN. Consequently, in this study, because of early metabolic and renal changes, treatment of young obese ZSF1 rats with rosiglitazone or enalapril should be considered early therapeutic rather than preventive treatments. The early development of diabetes indicates a very short pre-diabetic stage in obese ZSF1 rats, and because the earliest age at which lean and obese animals can be phenotypically separated is 5 weeks, genotyping in the first 2–3 weeks of life may be required before pre-diabetic studies and preventive treatments are initiated.

Early development of diabetes, hyperlipidemia, and hypertension led to progressive renal disease including severe proteinuria (25-fold increase in albuminuria compared with lean littermates) and development of glomerulosclerosis by 32 weeks of age. Surprisingly, by that age, there was no reduction in renal excretory function in Ob-ZSF1 rats and compared with lean littermates (Ln-ZSF1 group), Ob-ZSF1 rats even tended to have increased GFR (inulin clearance). It seems that at this stage of renal disease (32 weeks of age), GFR is influenced more by hyperglycemia than by the degree of renal injury, including mild glomerulosclerosis, severe proteinuria, and glomerular and tubulointerstitial inflammation. In this regard, when phenotype and treatment effects on GFR were analyzed in relation to hyperglycemia (presence or absence of glucosuria and high or low HbA1c levels, a significant influence of hyperglycemia on GFR in Ob-ZFS1 and Ob-enalapril groups versus Ln-ZSF1 and Ob-rosiglitazone groups was observed, suggesting hyperglycemia-induced hyperfiltration. The effect of hyperglycemia on GFR was more obvious when the latter was normalized by brain weight; on comparison to Ln-ZSF1 rats, the Ob-ZSF1, Ob-enalapril and Ob-rosiglitazone groups showed increased GFR by 22·9, 34·8, and 9·2% respectively (Table 2). Persistent increase in GFR has been reported in the majority of rat strains with experimental diabetes (O'Donnell *et al.* 1988, Palm *et al.* 2001), and it is plausible that in Ob-ZSF1 rats, hyperglycemia-induced hyperfiltration may offset the decline in GFR due to incipient renal injury. Another factor that may play a role in development and maintenance of glomerular hyperfiltration in diabetic rats is protein intake; increased protein intake is associated with elevated RBF and GFR in both experimental animals and humans (Brenner *et al.* 1982, Woods 1993), and it is, therefore, possible that hyperphagia-induced increase in protein intake might contribute to persistent elevation of GFR in obese ZSF1

Table 6 Kidney, heart, and liver histopathology in 32-week-old lean (Ln-ZSF1) and obese (Ob-ZSF1) ZSF1 rats and in obese animals treated with enalapril or rosiglitazone for 24 weeks (Ob-ENP, Ob-RGTZ)

	Ln-ZSF1	Ob-ZSF1	Ob-ENP	Ob-RGTZ
n	10	10	9	10
Kidney				
Nephropathy				
Absent	10	1	–	–
Minimal	–	–	5	–
Mild	–	–	3	7
Moderate	–	3	1	3
Severe	–	6	–	–
Cortical tubular dilatation				
Absent	–	–	–	4
Minimal	–	–	–	–
Mild	10	–	2	4
Moderate	–	7	7	2
Severe	–	3	–	–
Hydronephrosis				
Absent	10	8	8	8
Minimal	–	–	1	–
Mild	–	–	–	2
Moderate	–	1	–	–
Severe	–	1	–	–
Pyelonephritis				
Absent	10	10	6	9
Minimal	–	–	1	–
Mild	–	–	1	–
Moderate	–	–	1	1
Cortical arterial medial hypertrophy/sclerosis				
Absent	10	10	–	10
Minimal	–	–	2**	–
Mild	–	–	6**	–
Moderate	–	–	1**	–
Heart				
Microvesicular steatosis				
Absent	10	5	5	9
Minimal	–	3	2	1
Mild	–	2	2	–
Myocarditis				
Absent	6	3	3	–
Minimal	4	3	4	1*
Mild	–	4	2	9*
Liver				
Diffuse centrilobular hepatocyte fat vacuolation				
Absent	10	10	9	7
Minimal	–	–	–	–
Mild	–	–	–	3
Diffuse hepatocyte fat vacuolation				
Absent	10	–	–	3
Mild	–	3	1	7
Moderate	–	6	7	–
Severe	–	1	1	–
Diffuse hepatocyte glycogen vacuolation				
Absent	–	10	–	7
Present	10	–	9	3
Hepatocyte hyaline droplets				
Absent	10	10	9	7
Minimal	–	–	–	2
Mild	–	–	–	1

Minimal, present at ~1–25% of tissue section area; mild, present at ~26–50% of tissue section area; moderate, present at ~51–75% of tissue section area; severe, present at ~76–100% of tissue section area (*$P<0{\cdot}05$ versus Ln-ZSF1; **$P<0{\cdot}002$ versus all other groups).

rats, as in our Ob-ZSF1 group. This notion is further supported by the fact that treatment with rosiglitazone (which was associated with near normalization of food intake and glucose control and dramatic reductions in proteinuria, glomerulosclerosis, and tubulointerstitial inflammation) did not have a significant influence on GFR, and the Ob-rosiglitazone group even tended to have lower GFR than non-treated Ob-ZSF1 controls.

The above discussion raises questions concerning the similarity of renal changes in ZSF1 rats to those observed in human DN. Recently, validation criteria for murine models of progressive DN that replicate various features of human disease have been proposed by the NIH-created Animal Models of Diabetic Complications Consortium (Brosius *et al.* 2009). The criteria include i) >50% decline in GFR over the lifetime of the animal; ii) >10-fold increase in albuminuria compared to controls; iii) advanced mesangial expansion with or without nodular sclerosis and mesangiolysis; iv) any degree of arteriolar hyalinosis; v) glomerular basement membrane thickening by >50% over baseline; and vi) tubulointerstitial fibrosis. Although in this study Ob-ZSF1 rats at 32 weeks of age had similar GFR to that of lean littermates, we previously reported GFR in 44-week-old obese ZSF1 rats to be $1{\cdot}15 \pm 0{\cdot}21$, or $0{\cdot}33 \pm 0{\cdot}06$ ml/min per g kidney; this amounts to an age-related reduction of GFR of more than 50%. Similarly, we demonstrated 10- to 25-fold increase in proteinuria with significant mesangial expansion and tubulointerstitial fibrosis in animals older than 40 weeks (Tofovic *et al.* 2000, 2002, Zhang *et al.* 2007) and doubling of glomerular basement membrane thickness by 20 weeks of age in obese ZSF1 rats has been reported (Prabhakar *et al.* 2007). Recently, a combined classification for human type-1 and type-2 DN was developed that includes four hierarchical categories of glomerular lesions with separate evaluation for degrees of interstitial and vascular involvement (Tervaert *et al.* 2010); according to these criteria, 20- to 44-week-old obese ZSF1 rats develop class II glomerular lesions characterized by basement membrane thickening and mesangial expansion with mild to moderate (5–30%) glomerulosclerosis (Tofovic *et al.* 2000, 2002, Griffin *et al.* 2007, Prabhakar *et al.* 2007, Zhang *et al.* 2007, Rafikova *et al.* 2008, Joshi *et al.* 2009). Importantly, our previous studies indicate that by 50 weeks of age, male obese ZSF1 rats had >50% glomerulosclerosis (Tofovic *et al.* 2000, 2007). Similarly, obese animals develop interstitial and vascular lesions typical of human DN (Prabhakar *et al.* 2007) including interstitial fibrosis and tubular dilatation and atrophy, interstitial inflammation, and arteriolar thickening. This study and previous reports by us and others (Tofovic *et al.* 2000, 2002, 2007, Griffin *et al.* 2007, Prabhakar *et al.* 2007, Zhang *et al.* 2007, Rafikova *et al.* 2008, Joshi *et al.* 2009) thus indicate that the ZSF1 rat meets the criteria for progressive experimental diabetic renal disease and suggest that the obese ZSF1 rat may be the best available rat model for close simulation of human DN.

Early, long-term treatment with the PPARγ agonist rosiglitazone had remarkable metabolic effects: rosiglitazone nearly normalized blood glucose and HbA1c levels, reduced plasma lipids and food consumption to control values of lean littermates, and eliminated polyuria and polydipsia. The beneficial metabolic effects of rosiglitazone were associated with massive (+70%) increase in body weight;

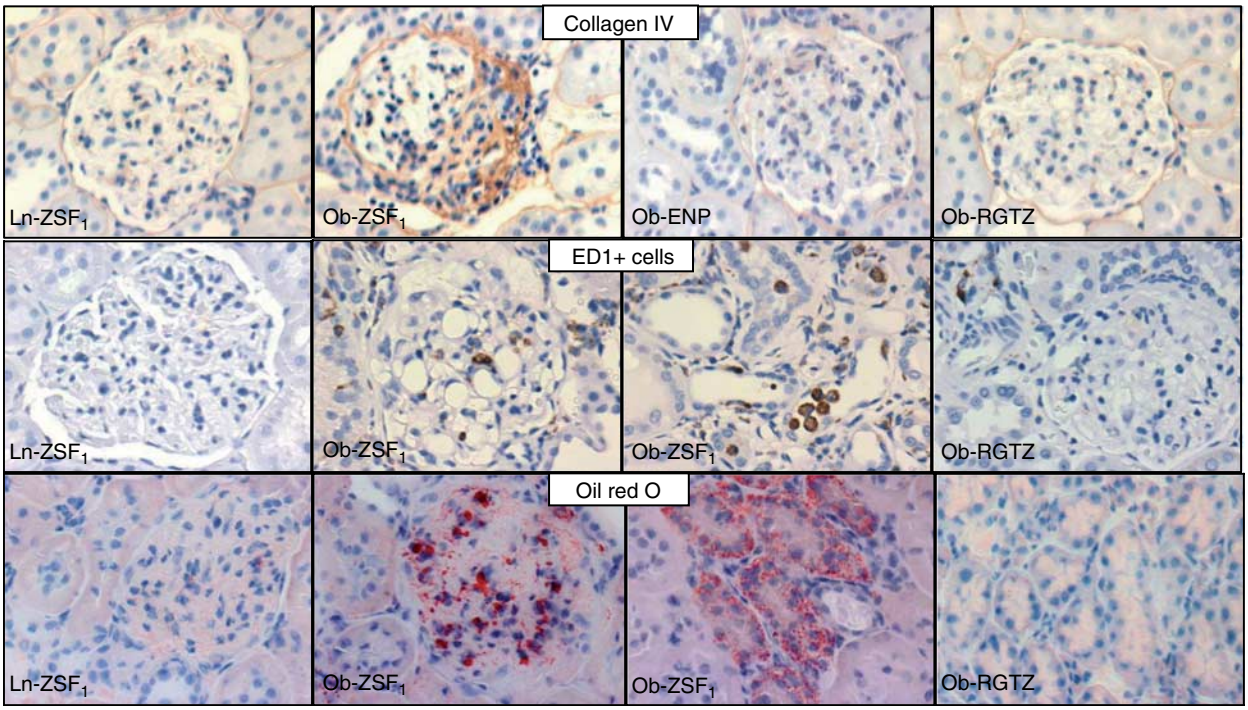

Figure 7 Immunohistochemistry of the obese ZSF1 rat kidney showing increased presence of collagen IV in a 32-week-old untreated obese ZSF1 rat compared with lean control and enalapril- and rosiglitazone-treated obese rats (top photomicrographs); macrophage (ED1+ cell) infiltration in glomeruli and tubulointerstitium in an obese ZSF1 rat, and absence of inflammatory cells in glomeruli of lean and rosiglitazone-treated obese rats (middle photomicrographs); significant neutral lipid accumulation in glomeruli and tubulointerstitium in Ob-ZSF1 rats, but minimal glomerular and tubulointerstitial lipid accumulation in lean control and rosiglitazone-treated obese rat (bottom photomicrographs).

Ob-rosiglitazone rats were morbidly obese, the body weights of some exceeding 1·2 kg. This finding is consistent with the well-defined effects of PPARγ agonist on adipocytes and abdominal adipose tissue redistribution (Chawla *et al.* 1994, Kelly *et al.* 1999). Similar to this study, in ZDF rats, short-term treatments with rosiglitazone (5–11 weeks) increase body weight by 40–50% while reducing food intake (Banz *et al.* 2007, Shoghi *et al.* 2009). The weight gain is largely due to increased body fat (Banz *et al.* 2007) and is associated with increased expression of genes encoding GLUT4, fatty acid synthase, and lipoprotein lipase, and also with improved glucose uptake and utilization and decreased fatty acid utilization and oxidation (Shoghi *et al.* 2009). These changes are consistent with reduced blood glucose levels, elimination of glucosuria, and augmented fat tissue formation.

Surprisingly, despite producing severe obesity, rosiglitazone markedly reduced renal deposition of neutral lipid and hepatocyte fat vacuolation. One recent report suggests that rosiglitazone may have tissue-specific effects on fat distribution by regulating the expression of both lipid storage and energy expenditure genes (Kang *et al.* 2010); this may explain the inhibitory effects of rosiglitazone on lipid accumulation in kidney and liver. Rosiglitazone attenuated elevated blood pressure and markedly reduced albuminuria, glomerular collagen IV expression, glomerular and interstitial inflammation, and glomerulosclerosis. Several mutually non-exclusive actions of rosiglitazone may contribute to this remarkable renal protection. The improved glycemic control undoubtedly plays a significant role, as hyperglycemia exerts multiple adverse effects on the kidney (Larkins & Dunlop 1992). The obese ZSF1 rat exhibits severe elevated cholesterol and triglycerides, and hyperlipidemia is considered a critical triggering factor for podocyte damage and subsequent glomerulosclerosis in obese Zucker rats (Coimbra *et al.* 2000). Therefore, the lipid-lowering effects of rosiglitazone may contribute to reduced renal injury. In addition, in recent years, it has become evident that rosiglitazone has direct renal protective effects independent of its effects on insulin resistance and glycemic control (Guan & Breyer 2001). In this regard, rosiglitazone reduces proteinuria and glomerulosclerosis in non-diabetic rat and mouse models of kidney disease (Bae *et al.* 2010, Liu *et al.* 2010); furthermore, in uninephrectomized type-2 diabetic *db/db* mice, a model of accelerated DN, the renoprotective effects of rosiglitazone seem to be independent of its effects on hyperglycemia. Both rosiglitazone and metformin exert similar levels of glucose control, but rosiglitazone-treated mice have lower serum creatinine and albuminuria, less severe glomerulosclerosis and tubulointerstitial injury, and fewer infiltrating macrophages compared with mice treated with

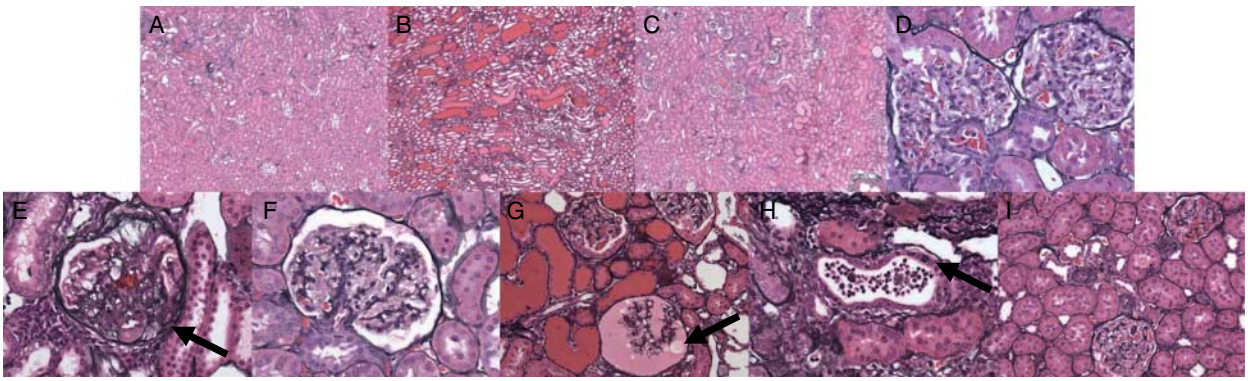

Figure 8 Photomicrographs of kidneys (methenamine silver-trichrome stain) in 32-week-old lean and obese ZSF1 rats and in 32-week-old obese animals treated with rosiglitazone or enalapril for 24 weeks; (A) lean control renal cortex with no histological abnormality (40×). (B) Renal medulla in Ob-ZSF1 rats showing distended collecting tubules by proteinaceous casts and no parenchymal chronic change (40×). (C) Ob-rosiglitazone rat renal cortex showing a small number of tubules distended by proteinaceous casts and no parenchymal chronic change (40×). (D) Normal glomeruli and adjacent tubules in lean control (400×). (E) Ob-ZSF1 rat glomerulus with multisegmental glomerulosclerosis with focal hyalinosis and an adhesion to Bowman's capsule (400×). (F) Normal glomerulus in Ob-rosiglitazone rat (400×). (G) Two glomeruli in Ob-ZSF1 rat with proximal tubular epithelium lining the lower portions of Bowman's capsule (tubularization of parietal epithelium versus take-off of proximal tubules) and one glomerulus with proteinaceous fluid distending Bowman's space. The glomerular tufts are normal and many of the adjacent cortical tubular profiles are distended by proteinaceous casts (200×). (H) Renal cortical proximal tubules in Ob-ZSF1 rats. The tubule in the center is injured with flattened epithelial cells, one with a mitotic figure (arrow) and variably degenerated cells in the lumen (400×). (I) Renal cortex with normal glomeruli and tubules in Ob-enalapril rat. The arterioles show marked concentric mural thickening and luminal narrowing. There is no evidence of ischemic injury (200×).

metformin (Tang *et al.* 2010). In rats with STZ-induced diabetes, rosiglitazone has no effects on hyperglycemia, glomerulosclerosis, or basement membrane thickening, but reduces albuminuria and renal macrophage infiltration (Setti *et al.* 2010). Thus, the anti-inflammatory effects of rosiglitazone may contribute to the marked renal protection in the current study. Finally, PPARγ is constitutively expressed in glomeruli, particularly in mesangial cells (Nicholas *et al.* 2001), and PPARγ agonists reduce mesangial cell collagen production (Routh *et al.* 2002). Likewise, in this study, rosiglitazone reduced intraglomerular collagen IV expression and glomerular and tubulointerstitial fibrosis.

The overall beneficial action of PPARγ agonists on insulin sensitization and their favorable metabolic and renal effects are confounded by unwanted weight gain (due to fat tissue accumulation and by potential adverse cardiac effects). Recent analysis of clinical studies suggests increased risk for development of heart failure in diabetic patients treated with rosiglitazone (Komajda *et al.* 2010, Sarafidis *et al.* 2010). In this study, untreated Ob-ZSF1 rats had minimal to mild myocarditis, and rosiglitazone only worsened cardiomyopathy in obese animals; significant differences in cardiac histopathology were found only between Ln-ZSF1 and Ob-rosiglitazone groups, but not between Ob-ZSF1 and Ob-rosiglitazone groups. It is not clear whether the remarkably beneficial metabolic effects of rosiglitazone may offset some of its adverse cardiac effects in obese ZSF1 rats, a model of diabetes with a genetic predisposition for heart failure. As with a study on spontaneously hypertensive rats (Wu *et al.* 2004), rosiglitazone was observed to reduce blood pressure in this study, and also to induce marked cardiac hypertrophy (35% increase in heart weight). Notably, direct hypertrophic effects of rosiglitazone through interaction with growth-promoting signaling pathways have been suggested (Bell & McDermott 2005, Festuccia *et al.* 2009).

Long-term treatment with high-dose enalapril normalized blood pressure, prevented albuminuria, and eliminated interstitial fibrosis and glomerulosclerosis. The only histological alteration in the Ob-enalapril group was mild to moderate tubular dilatation that was also seen in both lean and obese 8-week-old controls; therefore, this alteration cannot be attributed to enalapril treatment. Renal histopathology in enalapril-treated animals was nearly normal, except for somewhat surprising significant arteriolar medial hypertrophy. However, in our most recent study on obese ZSF1 rats (Tofovic, unpublished data), we detected similar renal vascular changes after 12 weeks of treatment with a high 60 mg/kg dose of enalapril, but not with doses of 3 or 10 mg/kg. It is noteworthy that similar vascular changes, including hypertrophy, hyperplasia, increased granularity (renin) of the juxtaglomerular apparatus, or exaggerated renin immunohistochemical staining have been previously described under experimental conditions where there is stimulation of the renin–angiotensin system or increased demand for renin, such as high-dose toxicity studies with a wide range of ACE inhibitors and angiotensin receptor blockers in rats, mice, dogs, and primates (Greaves 2000). Therefore, the observed arteriolar medial hypertrophy/vasoconstriction with high-dose enalapril is most likely an adaptive response to excessive doses of ACE inhibitor. The use of a supra-maximal dose of enalapril raises questions about the clinical relevance of the detected renal effects (near elimination of albuminuria and prevention of glomerulosclerosis). Although beneficial renal

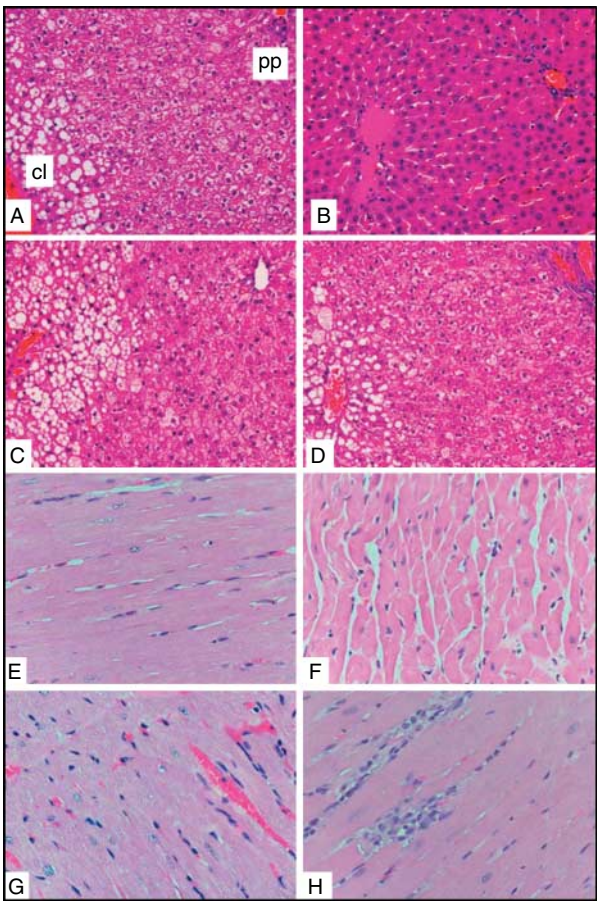

Figure 9 Photomicrographs of liver (A–D) and heart (E–H) of 32-week-old ZSF1 rats; (A) Ob-ZSF1 rat showing moderate diffuse hepatocellular steatosis focused primarily in the centrilobular (CL) areas and less so in periportal areas (PP); (B) absence of hepatocellular steatosis in Ln-ZSF1 rats. (C and D) Ob-enalapril and Ob-rosiglitazone rats showing moderate and mild hepatocellular steatosis respectively. Rosiglitazone treatment substantially reduced liver fat indicating a tendency toward a lean ZSF1 phenotype; (E) Ob-ZSF1 rat showing minimal diffuse myocellular vacuolation characteristic of lipid, (F) Ln-ZSF1 control with no vacuolation present; (G) Ob-enalapril rat showing mild myocellular vacuolation; (H) Ob-rosiglitazone rat with no myocellular vacuolation present. (Hematoxylin and eosin stain (A–D), 20× objective lens magnification; (E–H), 40× objective lens magnification).

effects of the observed magnitude should likely not be expected in humans, numerous studies on patients with both diabetic and non-diabetic chronic kidney disease suggest that supra-maximal doses of renin–angiotensin system inhibitors may provide additional renal protection over the doses used to reduce blood pressure (Leoncini *et al.* 2010).

In summary, this study indicates that, despite some limitations of the ZSF1 rat model, it is still the most appropriate rat model of DN, manifesting all of the features of human disease recommended by the Animal Models of Diabetic Complications Consortium (Fioretto *et al.* 2008). One of the limitations of this model is its persistently stable GFR, which does not parallel the progression of kidney injury in adult animals up to 32 weeks of age. This is most likely the result of persistent hyperglycemia and/or high protein intake. Therefore, longer studies that include animals aged 44–52 weeks may be required to evaluate the role of any intervention on renal excretory function. Rosiglitazone demonstrated a profound effect in this model, which may have resulted from reduced hyperglycemia, reduced protein intake, reduced lipid load, reduced blood pressure, or any combination of these factors. The model also demonstrated some of the cardiac side effects observed clinically with rosiglitazone. High-dose enalapril demonstrated blood pressure normalizing effects and nearly prevented diabetic kidney injury. We conclude that ZSF1 model is able to detect the clinical observations seen with rosiglitazone and enalapril in terms of primary endpoints and the secondary endpoints of renal and cardiac effects.

Declaration of interest

H B J, R M M, and S M P are employed by AstraZeneca Pharmaceuticals. B Z is employed by Bristol-Myers Squibb R&D, Princeton, NJ, USA.

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