Studies on kidney pathophysiological analyses in SDT fatty rat, a novel obese diabetic model

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Contents

List of Abbreviations

Abbreviation	Term
BUN	Blood urea nitrogen
Ccr	Creatinine clearance
CKD	Chronic kidney disease
DSS	Dahl salt-sensitive
eGFR	Estimated glomerular filtration rate
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ERG	Electroretinogram
ESRD	End stage renal disease
FFA	Free fatty acid
GFR	Glomerular filtration rate
GK	Goto Kakizaki
HbA1c	Hemoglobin A1c
HE	Hematoxylin and eosin
IDF	International Diabetes Federation
IENFD	Intraepidermal nerve fiber density
Kim-1	Kidney injury molecule-1
LDL	Low density lipoprotein
Lepr^{fa}	*fa* allele of the leptin receptor gene
LETO	Long-Evans Tokushima Otsuka

Abbreviation	Term
MNCV	Motor nerve conduction velocity
NGAL	Neutrophil gelatinase-associated lipocalin
NOD	Non-obese diabetes
OLETF	Otsuka Long-Evans Tokushima fatty
OP	Oscillatory potential
8-OHdG	8-Hydroxydeoxyguanosine
PAM	Periodic acid methenamine silver
PAS	Periodic acid Schiff
SBP	Systolic blood pressure
SD	Sprague-Dawley
SDT	Spontaneously Diabetic Torii
SGLT	Sodium glucose co-transporter
SNCV	Sensory nerve conduction velocity
STZ	Streptozotocin
TC	Total cholesterol
TG	Triglyceride
UAE	Urinary albumin excretion
UV	Urinary volume
VEGF	Vascular endothelial growth factor
ZDF	Zucker diabetic fatty

Chapter 1

General Introduction

Diabetic nephropathy is one of the microvascular complications induced by diabetes. Metabolic and hemodynamic abnormalities due to hyperglycemia cause a gradual reduction in renal function and urinary protein excretion. The growing population of patients with diabetes has resulted in an increase in the number of patients with diabetic nephropathy per year (Hall *et al.* 2003; Ritz & Stefanski 1996).

In 2002, the concept of chronic kidney disease (CKD) was proposed by the American Kidney Foundation, wherein performing a comprehensive assessment of renal disease and administering treatment aimed at early detection, healing and halting progression are considered important (National Kidney Foundation 2002). Recently, the number of patients with end-stage renal disease (ESRD) requiring dialysis and frequent transplants has increased. The increase in the number of such patients causes a significant medical issue financially. The prognosis of dialysis patients is extremely unfavorable and it is also becoming clear that CKD is not only a risk factor for progression to ESRD but is also a potent risk factor for the onset of cardiovascular disease.

CKD is considered to be a state of continuous deterioration of renal function, regardless of the disease background. In particular, diabetes is becoming re-recognized as an important underlying disease related to CKD. Currently, diabetic nephropathy is the main underlying disease in new patients on dialysis (United States Renal Data System 2013). Therefore, the treatment of diabetic nephropathy is an important issue for countermeasures against CKD. The pathogenesis of diabetic nephropathy is extremely

complicated. Besides diabetes and hypertension, multiple background factors, such as dyslipidemia, hyperinsulinemia and obesity, are thought to be involved in a complex manner in the development of diabetic nephropathy (The Diabetes Control and Complications Trial Research Group 1993; Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group 2003; UK Prospective Diabetes Study (UKPDS) Group 1998; Parving *et al*. 1983; Viberti & Wheeldon 2002; Parving *et al.* 2001; Sarafidis & Ruilope 2006; Trovati & Cavalot 2004; Inagi *et al.* 2001). Furthermore, these factors are assumed to induce blood circulation abnormalities, oxidative stress, inflammation or advanced glycation end-product formation, ultimately resulting in diabetic nephropathy due to the impairment of glomerular and tubulointerstitium in kidneys (Tan *et al.* 2007; Navarro-González & Mora-Fernández 2008; Forbes *et al.* 2008).

The treatment for diabetic nephropathy is mainly focused on the control of blood glucose and blood pressure. Strict control of blood glucose and blood pressure alleviates the rate of reduction in renal function and delays the introduction period for dialysis (The Diabetes Control and Complications Trial Research Group. 1993; Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. 2003; UK Prospective Diabetes Study (UKPDS) Group. 1998; Parving *et al.* 1983; Viberti & Wheeldon 2002; Parving *et al.* 2001). However, performing the dialysis still cannot be avoided. At present, effective therapeutic approaches for the treatment of diabetic nephropathy are lacking. Therefore, diabetic animal models play a critical role in the solution of several issues associated with diabetic nephropathy compared with animal models in other diseases.

Pathophysiological analyses of kidneys in diabetic animal models are becoming important for the development of novel therapies. At present, studies have been conducted using various diabetic animal models to overcome these issues. For example, a diabetic model obtained by destroying pancreatic β cells via administration of streptozotosin (STZ) and spontaneously diabetic models, such as non-obese diabetic (NOD) mice, KK-Ay mice, *Lep*^{db} (db/db) mice, Zucker diabetic fatty (ZDF) rats and Otsuka Long-Evans Tokushima fatty (OLETF) rats, are being used (Osorio *et al*. 2012; Katsuda *et al.* 2013, 2014).

The Spontaneously Diabetic Torii (SDT) fatty rat is an established congenic strain of an SDT rat with the *fa* allele of the leptin receptor gene (*Lepr*^{fa}). The *Lepr*^{fa} gene of the Zucker fatty rat was introduced into the genome of the SDT rat, an inbred model of non-obese type 2 diabetes mellitus, through the speed congenic method (Masuyama *et al*. 2005). The *fa* allele of the Zucker fatty rat is known to be a single recessive mutant gene in the leptin receptor and the introduction of the *fa* allele suppresses the signal transduction of leptin (Apweiler & Freund 1993). Zucker fatty rats that have abnormalities in leptin signaling and SDT fatty rats that have introduced *Lepr*^{fa} genes exhibit hyperphagia due to the inhibition of leptin-induced appetite suppression. By introducing *Lepr*^{fa}, rats exhibit obesity due to overeating. However, the difference in genetic background of rats influences the onset of diabetes. For example, Wistar fatty rats develop diabetes from 8 weeks of age, but Zucker fatty rats do not develop diabetes in spite of hyperinsulinemia and hyperlipidemia. The differing phenotype between Wistar fatty rats and Zucker fatty rats is suggested to be dependent on the vulnerability of the pancreas to hyperglycemia and hyperlipidemia via hyperphagia.

Hyperglycemia is an important pathogenic factor in the development of diabetic complications, such as microvascular and macrovascular diseases, in type 2 diabetes (Misra 2013). Hyperglycemia induces severe lesions in various organs, including the pancreas and kidney, which is well known as “glucotoxicity” (Odoni & Ritz 1999).

Hyperglycemia is attributed to two major defects, namely, insulin resistance and depletion of insulin secretion from the pancreas (Pfeifer *et al*. 1981; Taylor *et al*. 1994). The pancreatic β cells are key regulators of glucose homeostasis, and type 2 diabetes evolves due to an inability of islets to adapt the β cell mass to the increased insulin demand (Bonner-Weir *et al*. 2010; Karaca *et al*. 2009; Perl *et al*. 2010).

Pancreatic lesions in diabetic animal models are largely related to the incidence or development of diabetes mellitus. Pathological changes in islets, such as hypertrophy, atrophy, and fibrosis, are reportedly observed in obese type 2 diabetic rats. In humans, the relative β cell volume with both impaired fasting glucose and type 2 diabetes is decreased and the islet amyloid is increased (Butler *et al*. 2003). In SDT rats, similar pathological changes in the pancreas, such as atrophy and fibrosis, and a decrease in pancreatic content are observed (Mukai *et al.* 2014). Based on the vulnerability of pancreatic β cells, SDT rats show reduced insulin secretory function with age. SDT fatty rats, to which *Lepr*^{fa} genes are introduced into the genome of SDT rats, develop diabetes at younger ages compared with SDT rats (Matsui *et al*. 2008b; Ishii *et al*. 2010b). In addition to the early onset of diabetes, the incidence of diabetes in both male and female SDT fatty rats is 100%. SDT fatty rats are expected to be useful as an obese type 2 diabetes animal model. In terms of bleeding, SDT fatty rats had unpredictable factors, such as a decrease in the rate of pregnancy, during the early stage of the establishment

of the strain. However, in the course of strain maintenance, fertility was improved and a stable supply is now possible.

Basic biological analyses, such as blood and urine chemistry and histopathology of the pancreas, have been developed. Serum glucose levels in SDT fatty rats of both sexes were elevated from 6 weeks, and lipid parameters, such as serum triglyceride (TG) and total cholesterol (TC) levels, in the rats were elevated from 4 weeks of age. The observed hyperglycemia, hypertriglyceridemia and hypercholesterolemia were sustained for a long time afterwards (Ishii *et al*. 2010b; Matsui *et al*. 2008b). Male SDT fatty rats showed hyperinsulinemia from 4 to 8 weeks of age, but after 16 weeks insulin levels decreased to levels similar to those in SDT rats. In female rats, hyperinsulinemia was shown from 4 to 12 weeks of age, and insulin levels decreased gradually. In a glucose tolerance test conducted at 9 weeks of age, SDT fatty rats showed higher serum glucose levels after glucose loading, without any response to plasma insulin (Ishii *et al*. 2011b). Impaired glucose tolerance and insulin secretion deteriorated with aging. In pancreatic islets of female SDT fatty rats, pathological findings such as vacuolation, hypertrophy, and hemorrhage were observed from 8 weeks of age, and findings such as atrophy and fibrosis in islets were observed from 24 weeks of age (Ishii *et al*. 2010b). The hemorrhage in islets was specific to SDT fatty rats (Masuyama *et al*. 2004). In the pancreas of obese type 2 diabetic models, early changes such as islet hypertrophy and degranulation of β cells are caused by the development of hyperinsulinemia. Sustained hyperglycemia or development of diabetes induced degenerative changes, such as islet atrophy and fibrosis of islets with infiltration of inflammatory cells, and, finally, the islets were divided and replaced by connective tissues.

SDT fatty rats reportedly develop the three major diabetic complications, nephropathy, peripheral neuropathy and retinopathy (Matsui *et al*. 2008b; Ishii *et al.* 2010a). With early incidence of diabetes mellitus, diabetic nephropathy in SDT fatty rats was seen at younger ages than those in SDT rats. SDT fatty rats of both sexes show a remarkable rise in renal parameters, such as urine volume and urine protein, that were observed after 4 weeks of age (Matsui *et al*. 2008b). In male SDT fatty rats, histopathological examinations of the kidneys revealed changes in glomeruli from 16 weeks of age, and in renal tubules from 8 weeks of age (Matsui *et al*. 2008b). In the glomeruli, glomerulosclerosis was observed from 16 weeks of age, and sclerosis progressed with aging. Nodular lesions were observed at 40 weeks of age. In renal tubules, glycogen depositions in the tubular epithelium (Armanni-Ebstein lesions) and tubular dilation were noted from 8 weeks of age, and the change progressed from 8 to 16 weeks of age. In female SDT fatty rats, a qualitatively equal change was observed in histopathological findings of kidneys (Ishii *et al*. 2010b). The female rats revealed changes in glomeruli from 32 weeks of age, and in renal tubules from 16 weeks, and the changes progressed with aging. At 60 weeks of age, diffuse glomerulosclerosis, including increased mesangial matrix and glomerular hypertrophy, severely progressed in SDT fatty rats. Moreover, tubular and interstitial lesions, including fibrosis and inflammatory cell filtration, progressed in SDT fatty rats. There were no clear sex differences in the morphological characteristics of renal lesions.

In obese diabetic rats, albuminuria and proteinuria increased progressively with sustained hyperglycemia and aging. The increase in glomerular filtration rate (GFR) and renal hypertrophy were observed in the early stages of diabetic nephropathy. Glomerular pathological changes, such as diffuse glomerulosclerosis and nodular lesions, were

observed in obese diabetic rats. The expansion of nodular lesions to the mesangial matrix is characteristic of OLETF rats and SDT fatty rats. The tubulointerstitial scaring and inflammation were observed in ZDF rats and SDT fatty rats. Glomerular lesions, such as diffuse glomerulosclerosis and nodular lesions, and tubulointerstitial lesions, were also observed in human nephropathy (Calcutt *et al*. 2009). SDT fatty rats are considered to exhibit human-like characteristics of diabetic nephropathy. However, studies on the pathogenesis and mechanism of the development and progression of diabetic nephropathy, including early onset and drug responsiveness, in SDT fatty rats have not been performed in detail.

In the present studies, pathological analyses of kidneys were performed after unilateral nephrectomy or salt loading to elucidate the pathophysiological characteristics of the development and progression of diabetic nephropathy in the SDT fatty rat. Furthermore, the effects of hyperglycemia and hypertension on the development and progression of diabetic nephropathy in SDT fatty rats using pharmacological approaches were investigated.

Chapter 2

Effects of unilateral nephrectomy on renal function in male Spontaneously Diabetic Torii fatty rats: a novel obese type 2 diabetic model

INTRODUCTION

CKD is a worldwide public health problem associated with significant morbidity and mortality (Hoyert *et al*. 2006). Several risk factors contribute to the development and progression of CKD, including hypertension, diabetes, and dyslipidemia (Schaeffner *et al*. 2003; Agarwal 2009; Islam *et al.* 2009). In particular the increase in number of patients with obesity-associated type 2 diabetes has resulted in a rapid increase in patients who have ESRD and require dialytic life support (Ritz & Stefanski 1996; Hall *et al*. 2003). Despite efforts to develop means to prevent or arrest the progression of the disease, long-term prognosis of patients with established nephropathy remains poor (Petersen *et al*. 1988). Diabetic nephropathy has been recognized as a primary disease of CKD and the investigation of diabetic nephropathy is essential for the understanding of the pathogenesis of CKD.

Diabetic animal models have a critical role in the elucidation of mechanisms of diabetic complications and the development of novel drugs as treatments. Consequently, the understanding of the pathophysiology of renal lesions in diabetic models is beneficial in the design and development of therapies.

The SDT fatty rat, established by introducing the *fa* allele in Zucker fatty rats into the SDT rat genome, is a model for obese type 2 diabetes showing overt obesity, hyperglycemia, and hyperlipidemia from a younger age and resulting in early onset of

diabetic complications (Matsui *et al*. 2008a, 2008b; Morinaga *et al*. 2009; Ishii *et al*. 2010b; Katsuda *et al.* 2014). Furthermore, SDT fatty rats showed elevated blood pressure, in addition to the aforementioned metabolic abnormalities (Ishii *et al*. 2010a, 2011a). Therefore, the SDT fatty rat is considered to be a useful model for the analysis of diabetes and related complications such as diabetic nephropathy.

In this study, we investigated the effects of unilateral nephrectomy on renal function and morphology in SDT fatty rats.

MATERIALS AND METHODS

Animals

This experiment was conducted in compliance with the Guidelines for Animal Experimentation established for our biological/pharmacological laboratories. Male SDT fatty rats from Japan Tobacco colonies were used in this study. Animals were divided into 2 groups: those undergoing 1/2 nephrectomy (1/2 Nx group) or sham operation (Control group). Animals at 8 weeks of age underwent 1/2 Nx or sham surgery under anesthesia. A small lumbar incision was made, and the left kidney was removed. In sham-operated animals, the left kidney was exposed and gently manipulated but left intact. Animals were housed in suspended bracket cages and given a standard laboratory diet (CRF-1; Oriental Yeast, Tokyo, Japan) in a room with controlled temperature, humidity, and lighting.

Biophysiological Parameters

Body weight, biochemical parameters, and renal parameters were assessed from10 to 18 weeks of age, every 2 weeks. Blood samples were collected from the tail

vein of nonfasted rats. Serum glucose, TG, and TC were measured as a biochemical parameter using commercial kits (F. Hoffmann-La Roche, Basel, Switzerland) and an automatic analyzer (Hitachi, Tokyo, Japan).

Urinary volume (UV), urinary albumin, blood urea nitrogen (BUN), creatinine clearance (Ccr) and urinary biomarkers were evaluated as renal parameters. Urine samples were collected by placing the animals in metabolic cages with water for 6 h. Urinary albumin level was measured with a rat-albumin enzyme immunoassay (EIA) kit (Panapharm Laboratories, Kumamoto, Japan).Urinary creatinine, serum creatinine, and BUN were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi, Tokyo, Japan). Ccr was calculated by dividing the 6h urinary excretion of creatinine by serum creatinine level and body weight. Urinary biomarkers, such as kidney injury molecule-1 (Kim-1), Clusterin, Cystatin C and neutrophil gelatinase-associated lipocalin (NGAL), were measured using commercial kits (EMD Millipore, Darmstadt, Germany) and an automatic analyzer (BIO RAD, Hercules, USA).

Systolic blood pressure (SBP) and heart rate in a conscious non-fasted state were measured at 12 and 16 weeks of age by the indirect tail cuff method using a Softron BP-98A indirect blood pressure meter (Softron, Tokyo, Japan). Blood pressure and heart rate were measured between 13:00 and 16:00 hours. Five measurements were taken for each rat and subsequently averaged.

Histopathological Examination

Necropsy was performed at 18 weeks of age and kidneys were collected from all animals. Kidneys were weighed and fixed with 4% paraformaldehyde. After

resection, the tissues were paraffin-embedded using standard techniques and thin-sectioned (3–5 μm). Sections were stained with hematoxylin and eosin (HE) and periodic acid Schiff (PAS). Eight mice in the 1/2 Nx group and five mice in the Control group were examined histopathologically in a blinded manner.

Statistical Analysis

Results were expressed as means ± or + standard deviation. Statistical analyses of differences between mean values in the Control group and the 1/2 Nx group were performed using the F-test, followed by the Student's *t*-test or Aspin-Welch's *t*-test. Differences were defined as significant when $p < 0.05$.

RESULTS

Body Weight and Biochemical Parameters

Body weights in the 1/2 Nx group were comparable to those in the Control group from 10 to 18 weeks of age (1/2Nx group, 479.7 ± 116.2 g; Control group, 521.2 ± 40.3 g, at 18 weeks of age, respectively). The 1/2 Nx group and Control group had similar levels of plasma glucose from 10 to 18 weeks of age (1/2 Nx group, 751.0 ± 63 mg/dl; Control group, 799.9 ± 76.9 mg/dl, at 18 weeks of age, respectively). Serum TG levels in the 1/2 Nx group at 18 weeks of age were significantly higher than those in the Control group (1/2 Nx group, 501.9 ± 211.4 mg/dl; Control group, 309.6 ± 67.4 mg/dl, at 18 weeks of age, respectively). Serum TC levels tended to increase in the 1/2 Nx group (1/2 Nx group, 154.4 ± 51.7 mg/dl; Control group, 120.8 ± 18.7 mg/dl, at 18 weeks of age, respectively).

Renal Parameters

Urinary albumin excretion (UAE) in the 1/2 Nx group increased from 14 weeks of age compared with those in the Control group (Fig. 1A). Serum BUN levels in the 1/2 Nx group were significantly higher than those in the Control group during the experimental period (Fig. 1B). Kidney weights in the 1/2 Nx group were higher compared with those in the Control group (Fig. 1C). Ccr tended to decrease in the 1/2Nx group (1/2 Nx group, 0.23 ± 0.12 ml/h∗g; Control group, 0.31 ± 0.06 ml/h∗g, at 18 weeks of age, respectively). Urine volumes in the 1/2 Nx group were comparable to those in the Control group from 10 to 18 weeks of age (1/2 Nx group, 13.17 ± 6.58 ml; Control group, 15.90 ± 6.11 ml, at 18 weeks of age, respectively). Urinary biomarkers, such as Kim-1, Clusterin, Cystatin C and NGAL, in the 1/2 Nx group increased from 10 weeks of age compared with those in the Control group (Fig. 2).

Systolic Blood Pressure and Heart Rate

SBP levels at 12 and 16 weeks of age in the 1/2 Nx group were significantly elevated compared with those in the Control group (Fig. 3A). For heart rate, there were no differences among groups (Fig. 3B).

Histopathological Examinations in the Kidney

The results of histopathological examinations of the kidney at 18 weeks of age are shown in Table 1 and Fig. 4. The following findings in the glomerulus, tubule, and interstitium were observed in both the Control group and the 1/2 Nx group. Glomerulosclerosis was characterized by an increase in size of the glomerulus and diffuse thickening of the glomerulocapillary wall and the mesangial expansion, showing

partly segmental fibrosis in severe cases. Tubular lesions included tubular regeneration, dilatation, and hyaline casts, and interstitial lesions included fibrosis and inflammatory cell infiltration. The histological features of the kidney were not different between the 1/2 Nx group and the Control group; however, a more progressive pathological degree was observed in the 1/2 Nx group.

DISCUSSION

Glomerular lesions occurring due to renal mass reduction have been demonstrated (Steffes *et al*. 1978; O'Donnell *et al*. 1986). For example, kidney damage is exacerbated by nephrectomy in STZ diabetic rats (Lopes *et al.* 2004) and Zucker fatty rats (Kasiske *et al.* 1989). Therefore, unilateral nephrectomy is an effective method to accelerate the manifestation of renal alterations. In the present study, we evaluated the effects on renal function of SDT fatty rats subjected to unilateral nephrectomy, as well as the renal morphology of these animals.

Proteinuria, increased BUN, hypertension, and glomerular sclerosis, which are considered main characteristics of CKD, were observed in the 1/2 Nx group at a younger age compared with the Control group.

Compensatory hypertrophy of the remnant kidney after unilateral nephrectomy is well recognized (Orˇsi´c *et al*. 2011). This phenomenon is accompanied by pathological changes that lead to reduced renal function, although the weight of kidneys has not been used as an indicator of renal dysfunction (Orˇsi´c *et al*. 2011). In agreement with these findings, the remnant kidney in the 1/2 Nx group was significantly heavier than those in the Control group, and histopathological examinations of remnant kidneys in the 1/2 Nx group showed degenerative changes such as glomerulosclerosis in the

glomerulus and interstitial inflammation in the interstitium. These histopathological findings were not observed in normal Sprague Dawley (SD) rats.

Hyperglycemia is a known stimulus for renal hypertrophy and its association with reduction in renal mass affects this hypertrophy (Steffes *et al*. 1978; O'Donnell *et al*. 1986; Hostetter *et al*. 1981). In the present study, levels of plasma glucose were similar among groups. This result suggests that the contribution of hyperglycemia to the exacerbation of renal function in the 1/2 Nx group is likely low.

Altered lipid metabolism influences the development and progression of glomerular injury (Peric & Peric 1983; Kasiske *et al*. 1988). Lipid abnormalities in the 1/2 Nx group, which accompany a reduction in renal mass, may contribute to progressive glomerular damage.

Hypertension is a hemodynamic characteristic of CKD, which could accelerate the progression of renal dysfunction by worsening glomerular injury and proteinuria (Gagnon & Ansari 1990). It is possible that the increased blood pressure observed in the 1/2 Nx group in this study contributed to the accelerated glomerular injury and consequently led to marked proteinuria. Proteinuria has been considered a strong predictor of kidney disease outcome (Peterson *et al*. 1995). In addition, urinary biomarkers, such as Kim-1 and N-acetyl-β-D-glucosaminidase, have been reported (Luka *et al*. 2013; Nauta *et al*. 2011). Measuring these biomarkers may be useful to assess the severity of diabetic kidney damage.

In conclusion, induction of 1/2 Nx in SDT fatty rats led to functional and morphological damage of the remnant kidney and to hypertension, which are considered main characteristics of chronic kidney disease, at a younger age. The early onset of diabetic nephropathy in SDT fatty rats is an advantage for CKD research. The SDT fatty

rat has promise in the further elucidation of the pathogenesis of human diabetic nephropathy and in new drug discovery.

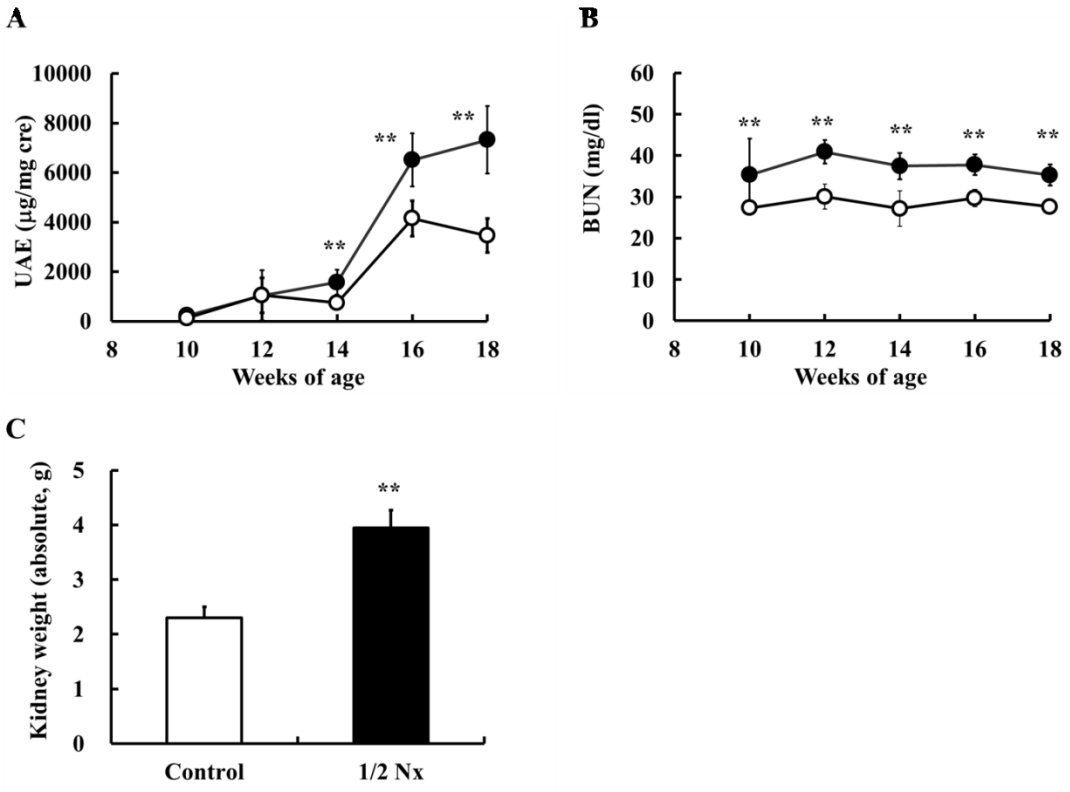

Figure 1 Changes in urinary albumin excretion (UAE) (A), blood urea nitrogen (BUN) level (B), and kidney weight (C) in the 1/2 Nx group and the Control group. White circles; Control group, black circles; 1/2 Nx group. Data shown as means ± or + standard deviation ((A) and (B) n = 8-10 and (C) n = 8-9). ** $p < 0.01$; significantly different from the Control group.

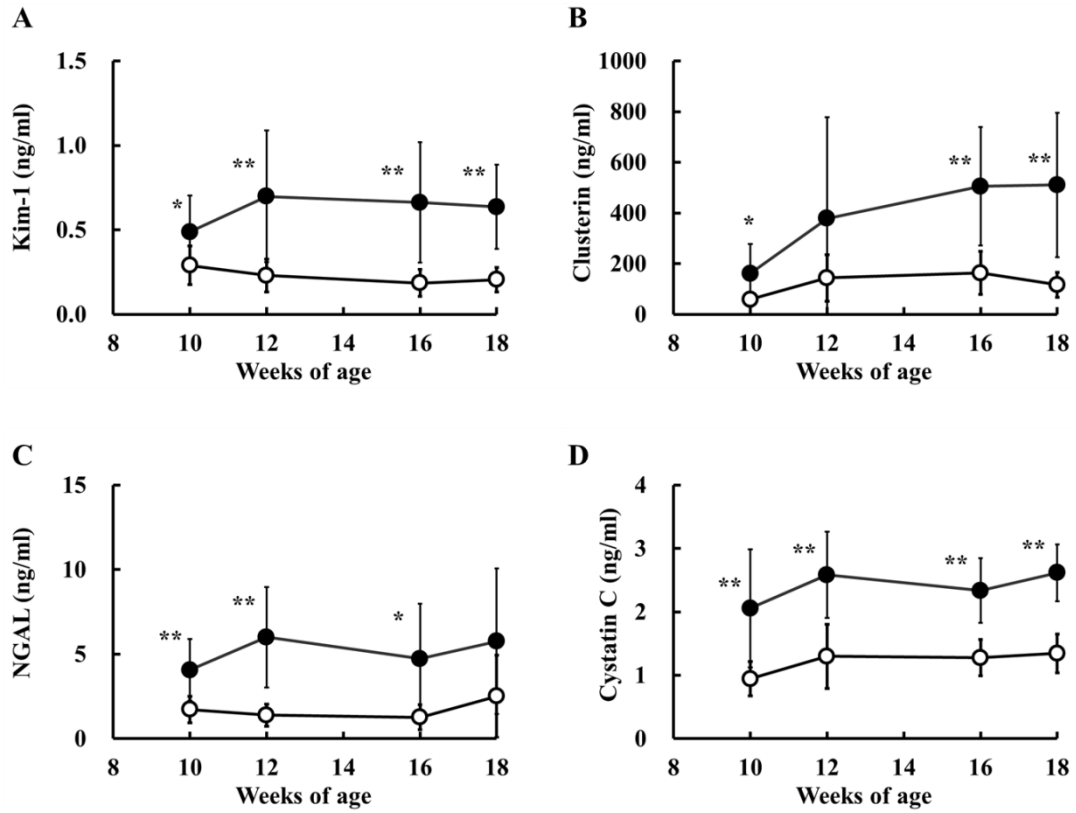

Figure 2 Changes in kidney injury molecule-1 (Kim-1) (A), Clusterin (B), neutrophil gelatinase-associated lipocalin (NGAL) (C), and Cystatin C (D) in the 1/2 Nx group and the Control group. White circles; Control group, black circles; 1/2 Nx group. Data shown as means ± standard deviation (n = 8-10). *$p < 0.05$, **$p < 0.01$; significantly different from the Control group.

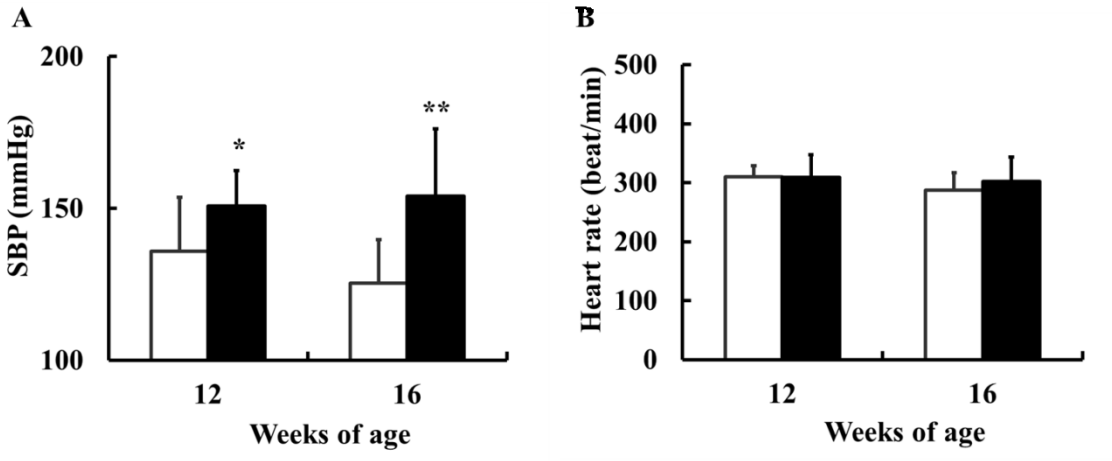

Figure 3 Systolic blood pressure (SBP) (A) and heart rate (B) at 12 and 16 weeks of age in the 1/2 Nx group and the Control group. White bars; SD group, black bars; 1/2 Nx group, gray bars. Data shown as means + standard deviation (n = 8-10). *$p < 0.05$, **$p < 0.01$; significantly different from the Control group.

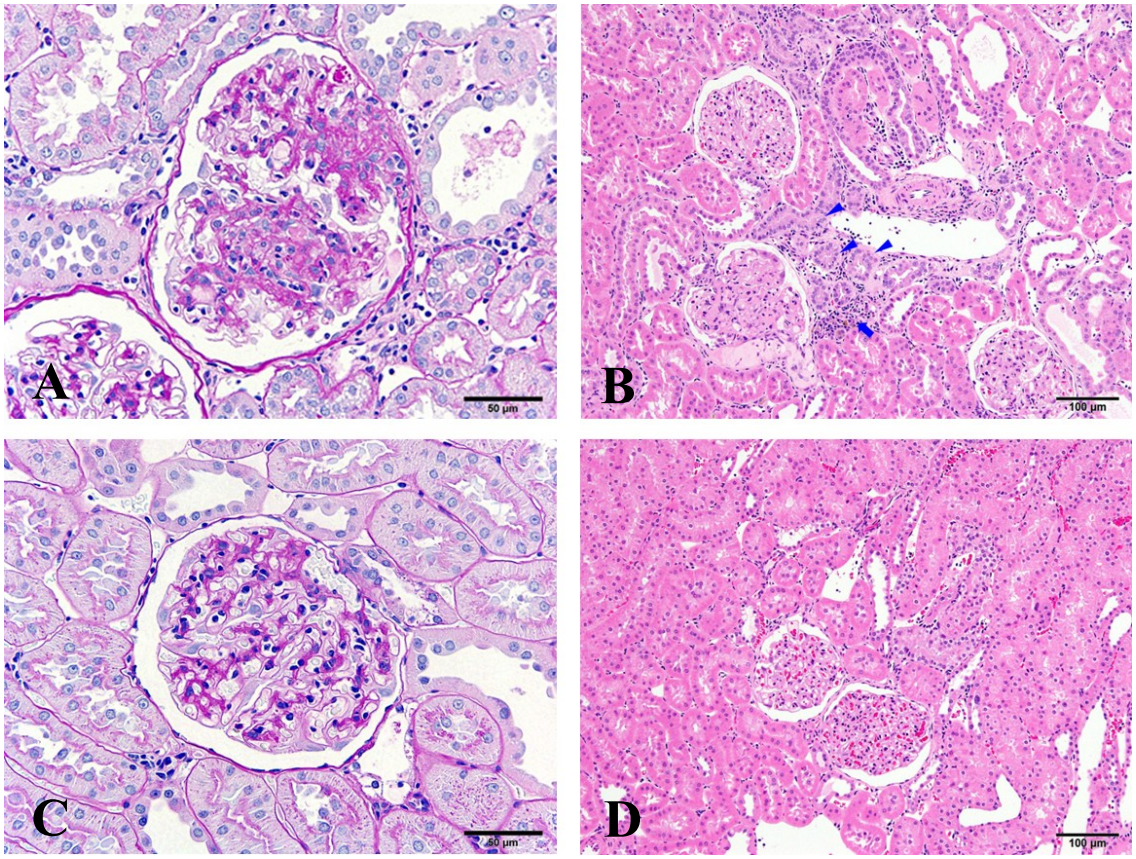

Figure 4 Photomicrograph of kidney tissues from the 1/2Nx group (A) and (B) and the Control group (C) and (D). Marked glomerulosclerosis (A), tubular regeneration (arrowhead in (B)), and inflammatory cell infiltration (arrow in (B)) were observed in the 1/2 Nx group. Periodic acid Schiff stain (PAS) stain ((A) and (C)), bar = 50 µm. Hematoxylin and eosin (HE) stain ((B) and (D)). Bar = 100 µm.

Table 1 Histopathological findings of kidney in the 1/2 Nx group and the Control group

Group	1/2 Nx								Control				
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Findings													
Glomerulus													
Glomerulosclerosis	+	+	2+	+	+	2+	+	+	±	±	±	±	-
Tubule													
Regeneration	+	2+	2+	2+	2+	2+	2+	2+	+	+	+	+	+
Dilatation	2+	2+	+	2+	+	2+	2+	2+	+	+	±	+	+
Hyaline cast	+	+	±	±	+	±	+	+	+	+	±	+	±
Deposit, hyaline droplet	±	-	-	-	-	-	-	-	-	-	-	-	-
Armanni-Ebstein change	+	+	+	+	+	±	±	-	+	+	+	+	+
Mineralization	-	+	+	+	±	+	+	+	+	+	+	-	+
Interstitial													
Fibrosis, interstitial	-	+	-	-	-	+	-	+	-	+	±	-	±
Infiltration, inflammatory cell, interstitial	+	+	2+	2+	+	2+	2+	2+	±	-	+	±	+

–: Negative, ±:Very slight, +: Slight, 2+: Moderate, 3+: Severe

Chapter 3

Physiological changes induced by salt intake in female Spontaneously Diabetic Torii-*Lepr*^{fa} (SDT fatty) rat, a novel obese diabetic model

INTRODUCTION

There is some evidence of a relationship between sodium and hypertension in patients with obesity and in those with diabetes mellitus (Ferrannini & DeFronzo 1989). In obesity, abnormalities in sympathetic nervous system activity and renin-aldosterone system can alter sodium balance. Moreover, changes in glucose and glucoregulatory hormones such as insulin and glucagon affect sodium metabolism. In diabetes mellitus, the above factors could be operative in addition to effects of renal lesions and vascular changes on blood pressure control (Tuck 1991). Dahl *et al*. (1958) reported that when sodium was restricted in obese hypertensive subjects, substantial reductions in blood pressure occurred. They proposed that sodium is an important factor in hypertension in obesity. Hypertension in type 2 diabetes is associated with various factors, including age, body weight, renal failure and essential hypertension (Simonson 1988; Sowers *et al.* 1988; Ferrannini & DeFronzo 1989), and relationships between sodium and insulin resistance or hyperinsulinemia are noticed. It was reported that high incidence of salt-sensitive blood pressure responses were observed in type 2 diabetes (Tuck 1991). Salt sensitivity often increases in patients with obesity or diabetes.

SDT fatty rat is a new model of obese type 2 diabetes, showing obesity, hyperglycemia and hyperlipidemia from a young age, and an early onset of diabetic complications (Matsui *et al*. 2008a, b; Morinaga *et al*. 2009; Ishii *et al*. 2010b).

Furthermore, SDT fatty rats showed an elevation of blood pressure, in addition to the above metabolic abnormalities (Ishii *et al*. 2010a, 2011a). In a previous study, we investigated physiological changes induced by salt intake using male SDT fatty rats (Ohta *et al.* 2014a). However, most lethal bodies were observed in salt-loaded male rats and we could not examine the pathological changes in the kidney.

In this study, we investigated physiological changes such as blood pressure and renal functions, including pathological analysis, using salt-loaded female SDT fatty rats.

MATERIALS AND METHODS

Animals

This experiment was conducted in compliance with the Guidelines for Animal Experimentation of our biological/ pharmacological research laboratories. Female SDT fatty rats from Japan Tobacco colonies were used in this study. Age matched SD rats were used as normal animals. SD rats were purchased from CLEA Japan Inc. (Tokyo, Japan). SDT fatty rats were given either distilled water (Control group) or 1% NaCl (NaCl group) in drinking water for 14 weeks, from 4 to 18 weeks of age. SD rats were given distilled water (SD group). Rats were housed in suspended bracket cages and given a standard laboratory diet (CRF-1; Oriental Yeast, Tokyo, Japan) in a room with controlled temperature, humidity and lighting.

Biophysiological Parameters

Body weight and biochemical parameters (glucose, insulin, TG, TC, BUN and creatinine levels) were periodically examined from 4 to 16 weeks of age. Food intake was examined from 8 to 16 weeks, every 4 weeks. Blood samples were collected from

the tail vein of non-fasted rats. Serum glucose, TG, TC, BUN and creatinine levels were measured using commercial kits (F. Hoffmann-La Roche, Basel, Switzerland) and an automatic analyzer (Hitachi, Tokyo, Japan). Serum insulin level was measured with a rat-insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan). Urine analysis, including UV, UAE and Ccr was performed at 12 and 16 weeks of age. Urine samples were collected by placing the animals in metabolic cages with water for 6 h. Urinary albumin level was measured with a rat-albumin EIA kit (Panapharm Laboratories, Kumamoto, Japan). Urinary creatinine levels were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi, Tokyo, Japan). Ccr was calculated by dividing the urinary creatinine excretion by the serum creatinine level and body weight. SBP and heart rate in the conscious non-fasted state were measured at 12 weeks of age by the indirect tail cuff method using a Softron BP-98A indirect blood pressure meter (Softron, Tokyo, Japan). The blood pressure and heart rate were measured between 13.00 and 16.00 hours. Five measurements were made for each rat and then averaged.

Histopathological Examination

Necropsy was conducted at 18 weeks of age and kidneys were collected from all the animals. The kidneys were weighed and fixed in 4% paraformaldehyde. After resection, the tissues were paraffin-embedded by standard techniques and thin-sectioned (3–5 μm). The sections were stained with HE, PAS, periodic acid methenamine silver (PAM), and Masson trichrome.

Statistical Analysis

Results were expressed as mean ± or + standard deviation. Statistical analysis of differences between mean values in the Control group and the NaCl group or the SD group was performed using the F-test, followed by Student's *t*-test or Aspin-Welch's *t*-test. Differences were defined as significant when $p < 0.05$.

RESULTS

Biophysiological Parameters

Changes in food intake and body weight are shown in Fig. 5. Food intake in the Control group increased or tended to increase as compared with that in the SD group and there were no significant differences in food intake between the Control group and the NaCl group during the experimental period (Fig. 5A). Body weights from 8 to 16 weeks of age in the Control group were higher than those in the SD group, and body weights in the NaCl group were comparable to those in the Control group (Fig. 5B). Changes in the biochemical parameters of glucose, TG, TC and insulin levels are shown in Fig. 6. The Control group showed hyperglycemia and hyperlipidemia, and there were no significant differences in blood glucose levels between the Control and the NaCl group, but the TG level in the NaCl group was decreased at 16 weeks of age (Figs. 6A and 6B). Serum TC levels in the NaCl group increased significantly as compared with those in the Control group (Fig. 6C). Serum insulin levels in the Control group increased or tended to increase as compared with those in the SD group, and there were no significant differences in insulin levels between the Control and the NaCl group (Fig. 6D).

Systolic Blood Pressure and Heart Rate

SBP levels at 12 and 16 weeks of age in the Control group were comparable to those in the SD group and SBP in the NaCl group was significantly elevated as compared with those in the Control group (SD group, 128.0 ± 17.5 mmHg; Control group, 127.4 ± 5.8 mmHg; NaCl group, 191.4 ± 25.4 mmHg, at 12 weeks of age, respectively) (Fig. 7A). Regarding heart rate, there were no differences among groups (Fig. 7B). Heart rates at 12 and 16 weeks of age in the Control group were lower than those in the SD group and heart rate at 16 weeks of age in the NaCl group was significantly increased as compared with that in the Control group (Fig. 7B).

Renal Parameters

Changes in renal parameters (serum BUN level, UV, UAE and Ccr) are shown in Fig. 8. Serum BUN levels in the Control group were comparable to those in the SD group at 12 and 16 weeks of age, but the levels in the NaCl group were decreased as compared with those in the Control group (Fig. 8A). UV in the Control group increased as compared with that in the SD group, but UV in the NaCl group tended to decrease at 16 weeks of age (Fig. 8B). UAE in the Control group increased as compared with those in the SD group, and those levels in the NaCl group were comparable to those in the Control group (Fig. 8C). Ccr at 16 weeks of age in the Control group was increased as compared with that in the SD group, but Ccr in the NaCl group was significantly decreased (SD group, 0.23 ± 0.06 ml/h*g; Control group, 0.40 ± 0.11 ml/h*g; NaCl group, 0.18 ± 0.08 ml/h*g, respectively) (Fig. 8D).

Histopathological Examination in Kidney

The results of histopathological examination are shown in Table 2 and Fig. 9. A

characteristic renal finding in NaCl group was a marked thickening of the walls of arterioles near the glomerulus. Some thickened arterioles were accompanied by fibrous and hyaline accumulation. The vascular changes were not obvious in the Control group. The following findings in the glomerulus, tubule and interstitium were observed in both the Control and NaCl groups. Glomerulosclerosis was characterized by slight increase in size of the glomerulus, and diffuse thickening of the glomerulocapillary wall and the mesangial expansion, showing partly segmental fibrosis in severe cases. Tubular lesions included tubular regeneration, dilatation, hyaline cast, vacuolation and deposit of hyaline droplets, and interstitial lesions included fibrosis and inflammatory cell infiltration. The severity of these findings was higher in the NaCl group than those in the Control group. These histological findings were not observed in the SD group. Kidney weight in the Control group increased as compared with that in the SD group, but there were no significant differences in kidney weights between the Control and the NaCl groups (data not shown).

DISCUSSION

Female SDT fatty rats showed abnormalities of renal parameters, such as increases of BUN, UV and urinary protein levels, and renal lesions such as glomerulosclerosis and nodular lesions (Ishii *et al*. 2010b). Also, female SDT fatty rats showed a temporary increase of blood pressure (Ishii *et al.* 2011a). In the present study, physiological changes in blood pressure and renal function were examined in female SDT fatty rats after salt loading.

In salt-loaded SDT fatty rats, there was a small decrease in food intake (Fig. 5A) and a tendency for decrease in blood glucose and TG levels was observed (Figs. 6A and

6B). Blood TC levels in salt-loaded SDT fatty rats were increased with aging (Fig. 6C). The reason for the increase in TC level after salt loading is unknown. It is reported that TC synthesis is decreased or unchanged after salt loading (Karkendall *et al*. 1976; Abe *et al.* 1986).

A significant elevation of SBP in salt-loaded SDT fatty rats was a remarkable result (Fig. 7A). In humans, the role of salt in the control of blood pressure is great in obesity and in diabetes mellitus. Strong relationships between hypertension and obesity or diabetes have been found in epidemiological studies regardless of age, race or gender (Kannel *et al*. 1967; Stamler *et al.* 1978). A salt-blood pressure relation also became clear in female SDT fatty rats in the present study. It is considered that one reason for the deterioration of renal function, such as a decrease of Ccr and pathological changes, in the salt-loaded SDT fatty rats is the hypertension induced by salt intake. In ZDF rats, SDT rats and SD rats, SBP was not increased by salt intake (data not shown). In other diabetic animal models, an increase of blood pressure after salt loading was reported in Goto Kakizaki (GK) rats, STZ rats, Zucker fatty rats and Wistar fatty rats (Reddy & Kotchen 1992; Suzuki *et al.* 1996; Carlson *et al.* 2000; Hayashida *et al.* 2001; Olearczyk *et al.* 2009; Osorio *et al.* 2009; Zhang *et al*. 2009). On the other hand, blood pressure after salt loading did not change in Long-Evans rats or neonatal STZ rats (Iwase *et al*. 1987; Sima *et al*. 2012). In SDT fatty rats at 12 weeks of age, SBP was increased from 127 mmHg to 191 mmHg by salt intake for 8 weeks (Fig. 7A). The degree of increase in blood pressure was large, as compared with other animal models, suggesting that SDT fatty rat has a noticeably high salt sensitivity. It is unclear what mechanisms account for this severe salt sensitivity in SDT fatty rats. Since heart rates in the NaCl group did not decrease (Fig. 7B), impaired baroreflex might contribute to

salt-induced hypertension (Bunag & Barringer 1988). Detailed studies for elucidation of the mechanisms such as vasodilatory capacity and sympathetic nervous system activity should be performed.

Blood BUN level is increased in renal disease, but the BUN levels in salt-loaded SDT fatty rats were decreased (Fig. 8A). The decrease of BUN levels in salt-loaded rats might be caused by an increase of plasma volume via salt intake. UV in the Control group was elevated as compared with the SD group, but the elevation tended to be suppressed in the NaCl group (Fig. 8B). The decrease in the NaCl group might also be introduced by suppression of hyperglycemia (Fig. 6A). Since Ccr in the NaCl group was decreased (Fig. 8D), glomerular filtration function in the SDT fatty rat might be deteriorated by persistent salt intake. In the histological examination of the kidney, a hypertension-related finding was observed as a marked thickening of arterioles. An animal with the highest value of SBP in the salt-loaded group showed the highest severity of thickened arteriole, in addition to the other renal findings. Glomerulosclerosis, tubular lesions and interstitial lesions in SDT fatty rats were deteriorated by salt intake (Fig. 9). Chronic salt intake is considered to have induced the renal dysfunction and the histological changes in these female SDT fatty rats. Similar renal lesions were also observed in Dahl salt-sensitive (DSS) rats. In DSS rats, progressive deterioration of renal structure and function occurs with aging. The aging kidney is characterized by a decrease in glomerular filtration rate and renal blood flow, decreased urine-concentrating ability, and increased glomerulosclerosis and tubulointerstitial fibrosis (Abrass *et al.* 1995; Maric *et al.* 2004).

In conclusion, SDT fatty rats showed salt-sensitive hypertension and deterioration of renal function and histology after salt loading. The SDT fatty rat is a

useful model for investigating the mechanism of high salt sensitivity in obesity and in diabetes mellitus.

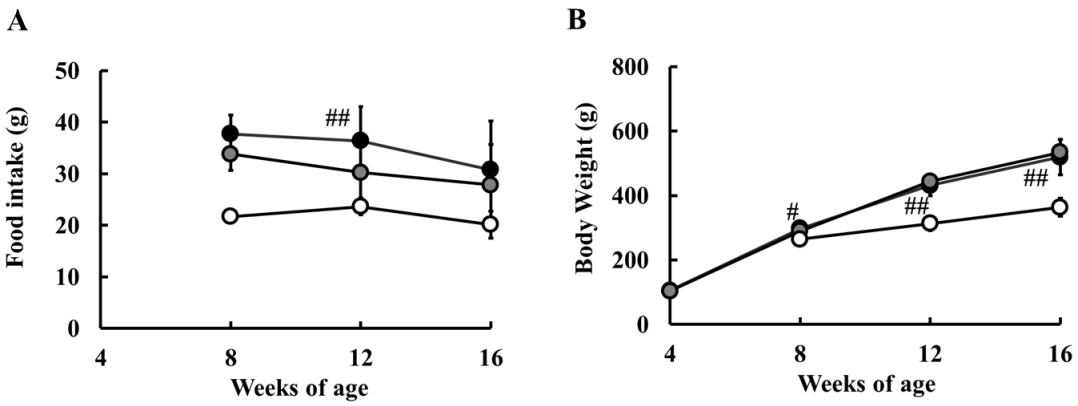

Figure 5 Changes in food intake (A) and body weight (B) in SDT fatty rats by salt intake. White circles; SD group, black circles; Control group, gray circles; NaCl group. Data represent mean ± standard deviation (n = 5). #$p < 0.05$, ##$p < 0.01$; significantly different from the SD group.

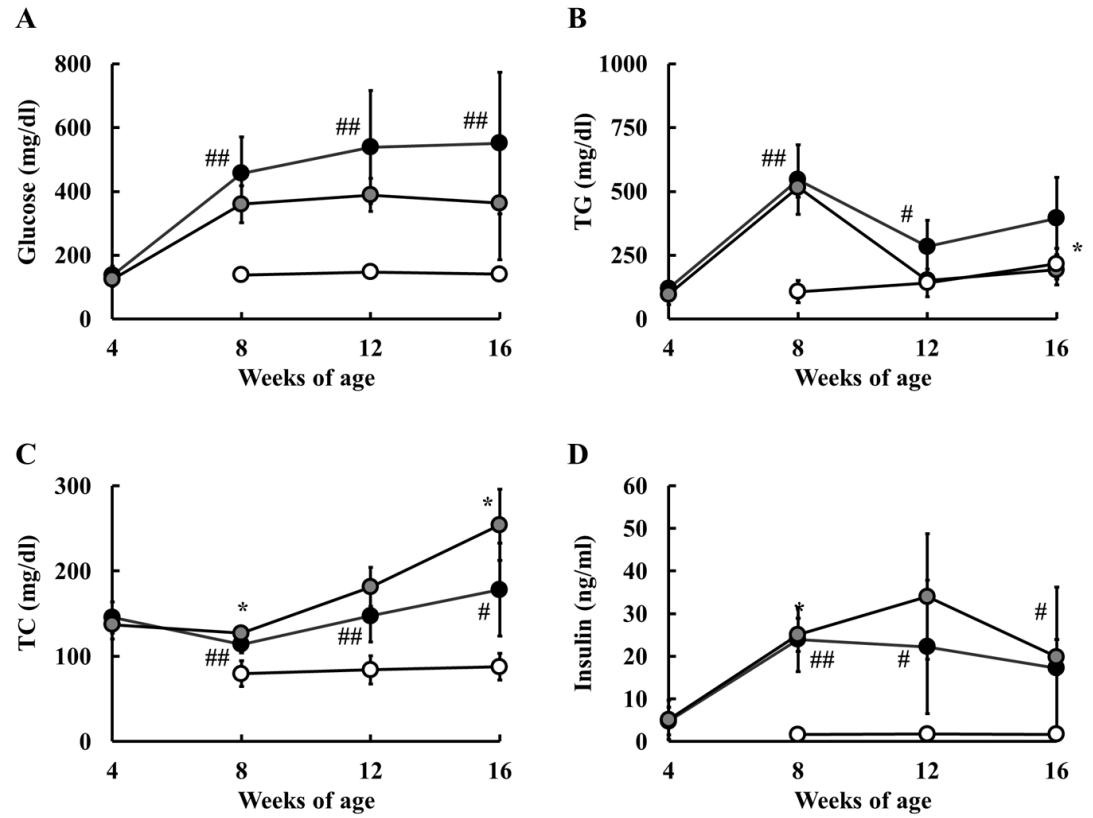

Figure 6 Changes in serum glucose (A), triglyceride (TG) (B), total cholesterol (TC) (C), and insulin (D) levels in SDT fatty rats by salt intake. White circles; SD group, black circles; Control group, gray circles; NaCl group. Data represent mean ± standard deviation (n = 5). *$p < 0.05$; significantly different from the Control group. #$p < 0.05$, ##$p < 0.01$; significantly different from the SD group.

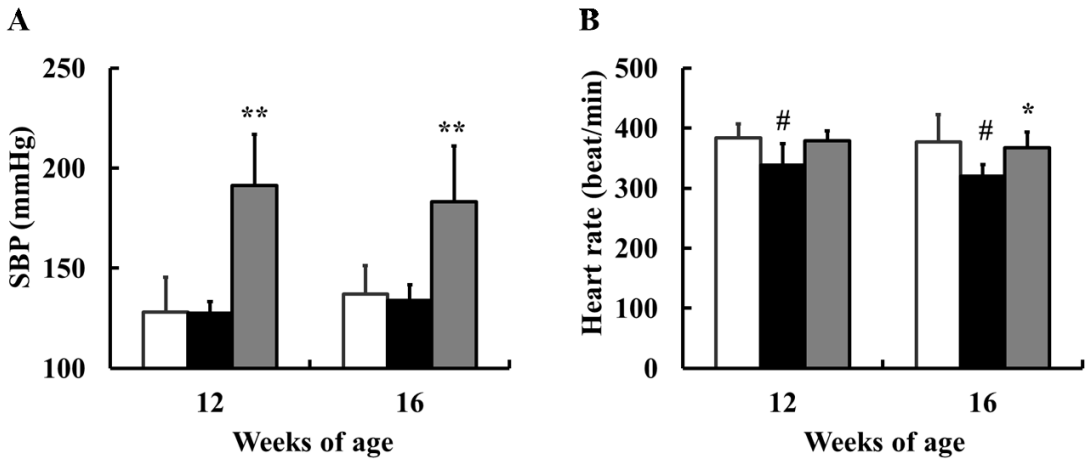

Figure 7 Systolic blood pressure (SBP) (A) and heart rate (B) at 12 and 16 weeks of age in salt-loaded SDT fatty rats. White bars; SD group, black bars; Control group, gray bars; NaCl group. Data represent mean + standard deviation (n = 5). *$p < 0.05$, **$p < 0.01$; significantly different from the Control group. #$p < 0.05$; significantly different from the SD group.

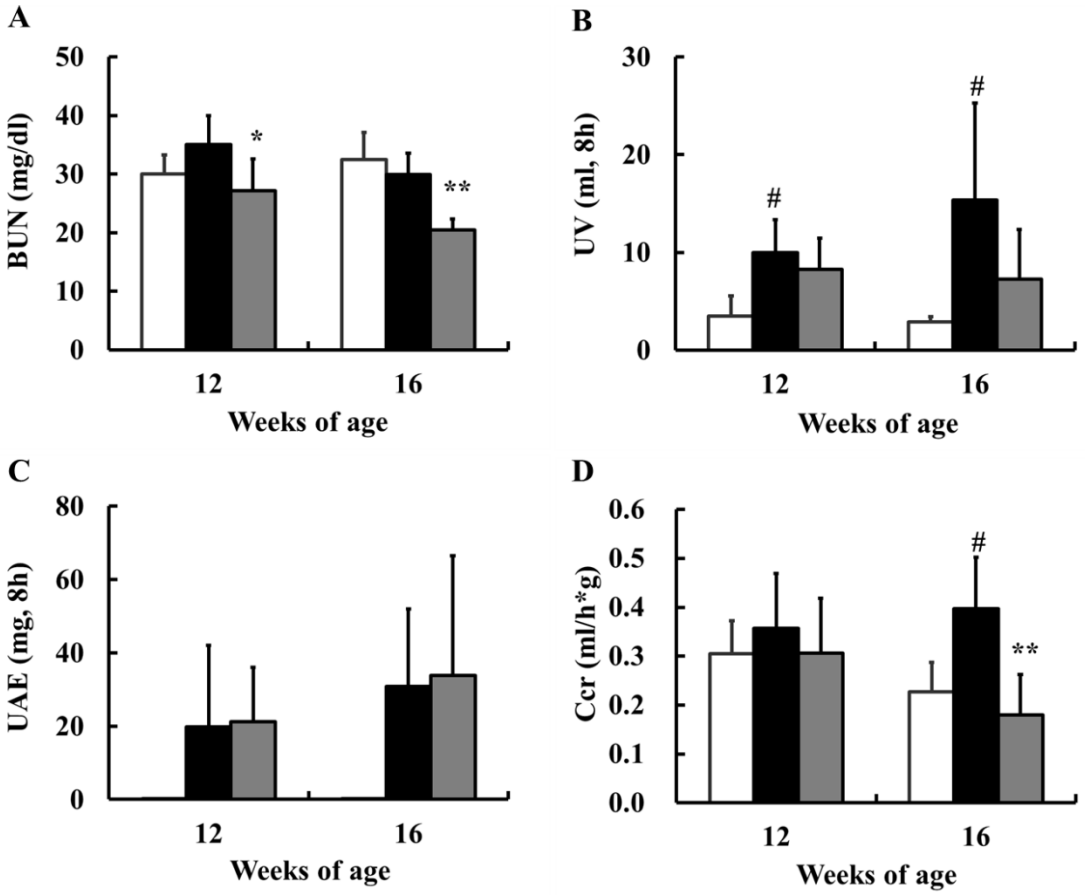

Figure 8 Changes in blood urea nitrogen (BUN) level (A), urinary volume (UV) (B), urinary albumin excretion (UAE) (C), and creatinine clearance (Ccr) (D) in SDT fatty rats by salt intake. White bars; SD group, black bars; Control group, gray bars; NaCl group. Data represent mean + standard deviation (n = 5). *$p < 0.05$, **$p < 0.01$; significantly different from the Control group. #$p < 0.05$; significantly different from the SD group.

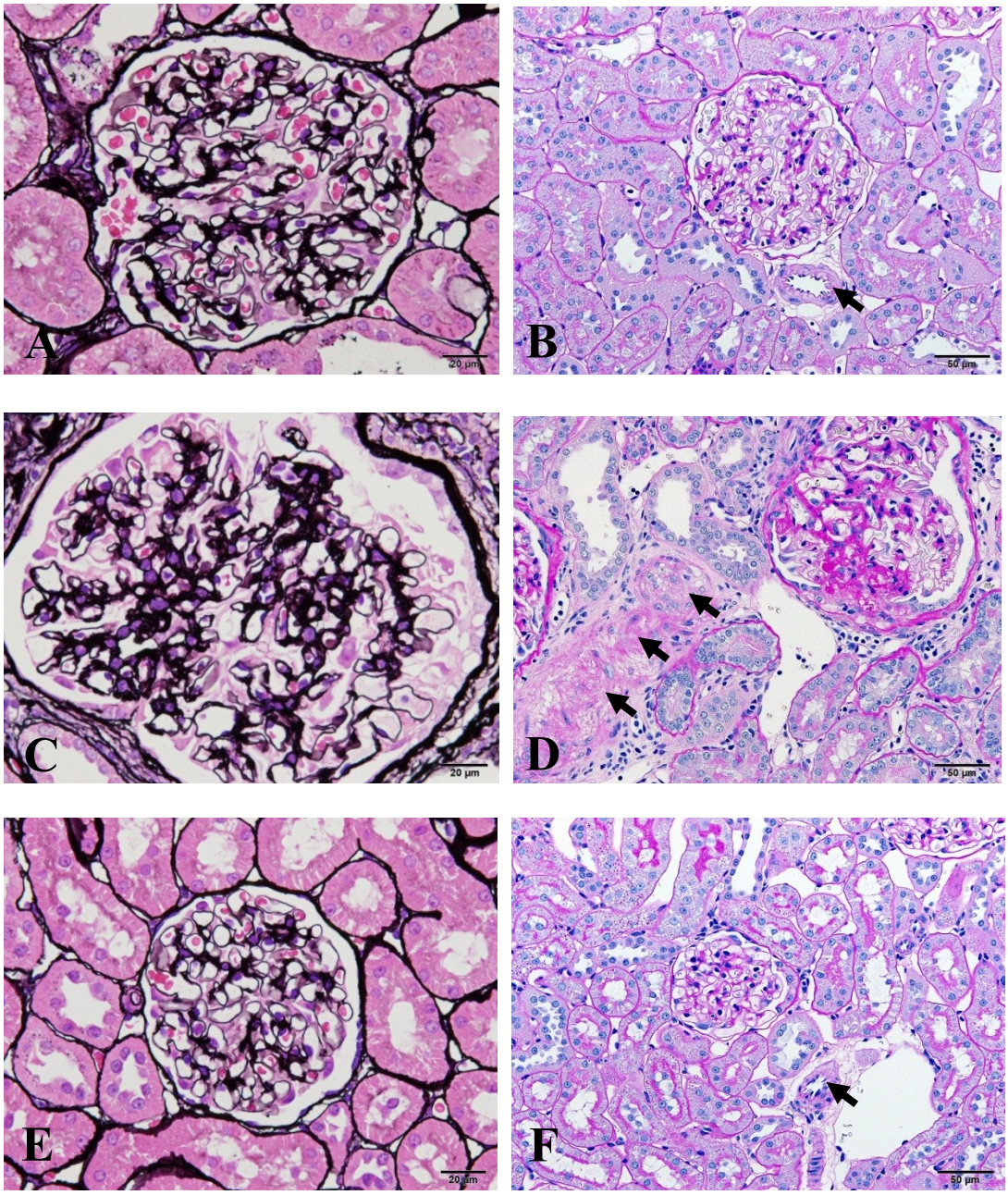

Figure 9 Photomicrographs of the kidney tissue from Control (A) (B), salt-loaded SDT fatty rats (C) (D) and SD rat (E) (F). Glomerulosclerosis is deteriorated in SDT fatty rats by salt intake (C and D). An arteriole near the glomerulus is markedly thickened (arrows in D). Normal arteriols (arrows in B and F). Periodic acid methenamine silver (PAM) stain (A, C and E). Bar = 20 μm. Periodic acid Schiff (PAS) stain (B, D and F). Bar = 50 μm.

Table 2 Histopathological findings of kidney in the Control group, the NaCl group, and the SD group

Group	Control					NaCl					SD				
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Findings															
Glomerulus															
Glomerulosclerosis	+	+	+	+	+	2+	+	+	2+	+	-	-	-	-	-
Tubule															
Regeneration	+	+	+	±	2+	2+	+	+	2+	+	-	-	-	-	-
Dilatation	+	+	-	+	-	2+	-	+	+	+	-	-	-	-	-
Hyaline cast	+	±	±	-	+	2+	+	±	2+	+	-	-	-	-	-
Vacuolation	+	±	+	-	-	+	+	+	+	+	-	-	-	-	-
Deposit, hyaline droplet	±	±	±	-	-	+	+	-	-	+	-	-	-	-	-
Armanni-Ebstein change	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
Mineralization	+	+	+	±	+	+	+	+	+	+	-	-	-	-	-
Interstitium															
Thickening, arteriole	-	-	-	-	-	3+	±	±	+	-	-	-	-	-	-
Fibrosis	±	±	±	±	+	2+	+	+	2+	+	-	-	-	-	-
Infiltration, inflammatory cell	+	±	+	±	+	2+	2+	+	2+	+	-	-	-	-	-

- : Negative, ± : Very slight, + : Slight, 2+ : Moderate, 3+ : Severe

Chapter 4

Contribution of hyperglycemia on diabetic complications in obese type 2 diabetic SDT fatty rats: effects of SGLT inhibitor phlorizin

INTRODUCTION

Diabetes mellitus is one of the most common metabolic disorders, and the number of diabetic patients has been increasing worldwide. The International Diabetes Federation (IDF) reported that the 366 million patients with diabetes in 2011 will increase to 552 million by 2030 (International Diabetes Federation 2011). Even worse, more than half of all diabetics have one or more diabetic microvascular complications, such as diabetic nephropathy, diabetic peripheral neuropathy or diabetic retinopathy, all of which seriously threaten quality of life. To clarify the pathogenetic mechanism of human diabetes and its complications, and to develop drugs for diabetes, experimental diabetic animal models play critical roles.

The SDT fatty rat is a new model for obese type 2 diabetes established by introducing the *fa* allele of the Zucker fatty rat into the original (non-obese) SDT rat genome to defect leptin receptor signaling. Since SDT fatty rats develop marked hyperglycemia with hyperinsulinemia, hyperlipidemia and hypertension shortly after weaning (Ishii *et al.* 2010a, 2010b, 2011a; Kemmochi *et al*. 2013; Masuyama *et al.* 2005; Ohta *et al*. 2014b). SDT fatty rats develop severe microvascular complications at an early age (Kemmochi *et al*. 2013; Masuyama *et al*. 2005; Matsui *et al*. 2008b). Therefore, this animal model is useful for investigating diabetic complications and for evaluating new drugs. Previously, we investigated diabetic microvascular complications in original SDT rats by controlling blood glucose level with insulin treatment and

showed that complications are caused by severe hyperglycemia (Sasase *et al*. 2006, 2009). However, because of hyperinsulinemia associated with marked insulin resistance, insulin treatment failed to control blood glucose level in SDT fatty rats (unpublished data). Therefore in the present study, we investigated diabetic complications by controlling blood glucose level with daily phlorizin treatment. Phlorizin is a natural compound originally isolated from apple trees (Ehrenkranz *et al*. 2005). Its pharmacological mechanism is inhibiting sodium glucose co-transporters (SGLTs) distributed in the proximal tubule brush border (SGLT2) and gastrointestinal tract (SGLT1), leading to renal glucosuria and blocking intestinal glucose absorption, both of which reduce hyperglycemia (Abdul-Ghani & DeFronzo 2008). In anticipation of these mechanisms, we administered phlorizin to SDT fatty rats to control blood glucose level and studied whether and how hyperglycemia causes diabetic microvascular complications in this model.

MATERIALS AND METHODS

Animals and Chemicals

Female SDT fatty rats from our colony were used in the study. At six weeks of age, SDT fatty rats were divided into two groups (n=8); a phlorizin-treated group and a vehicle treated group (Control group). Age-matched female SD rats (Charles River Laboratories Japan, Yokohama, Japan) were used as normal animals (SD group) (n=8). All animal protocols used in the study were in strict compliance with our own Laboratory Guidelines for Animal Experimentation. Animals were housed in a climate-controlled room (temperature 23 ± 3°C, humidity 55 ± 15%, 12 h lighting cycle) and allowed free access to basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and

water.

Phlorizin (Kanto chemical, Tokyo, Japan) was suspended in 20% propylene glycol and injected subcutaneously once daily (100 mg/kg/day) to animals in the phlorizin-treated group for 23 weeks. 20% propylene glycol was administered to animals in the Control group and SD group.

Biochemical Parameters

During the experimental period, biochemical parameters were monitored. Blood samples were collected from the tail vein under fed condition. Glucose, hemoglobin A1c (HbA1c), TG, free fatty acid (FFA) and TC were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi, Tokyo, Japan). Commercial ELISA kits were used to measure plasma insulin (Morinaga Institute of Biological Science, Yokohama, Japan).

Evaluation of Diabetic Nephropathy

Urine samples were collected for 24 hr using metabolic cages. During urine sampling, animals were not limited to access to diet and water. Urinary glucose level was measured as described above. Both urinary and plasma creatinine levels were measured with an automatic analyzer to calculate Ccr. Urinary albumin (Exocell, Philadelphia, USA.) and urinary 8-Hydroxydeoxyguanosine (8-OHdG) (Nikken SEIL, Shizuoka, Japan) were measured using commercial kits.

Evaluation of Peripheral Neuropathy

Nerve conduction velocity was measured in accordance with previously

described methods (Yamaguchi *et al*. 2012). Briefly, the sciatic nerve was stimulated at the sciatic notch and the Achilles tendon using adequate intensity under 37.5 mg/kg of sodium pentobarbital (Kanto chemical) and 3 mg/kg of diazepam anesthesia. Action potentials in the muscle were recorded via PowerLab through a needle electrode. Motor nerve conduction velocity (MNCV) was calculated from the delta latency between M-wave peaks divided by the distance of the nerve length measured. Sensory nerve conduction velocity (SNCV) was also calculated from F-wave peaks.

Evaluation of Retinopathy and Cataracts

Electroretinograms (ERGs) were performed as previously described (Sasase *et al*. 2006) with slight modifications. Briefly, rats were adapted to darkness for at least 60 min and anesthetized with an intraperitoneal injection of 37.5 mg/kg ketamine (Daiichi Sankyo Propharma, Tokyo, Japan) and 4.5 mg/kg xylazine hydrochloride (MP Biomedicals, Santa Ana, USA). A 40 J xenon lamp was flashed after pupillary mydriasis with 0.5% tropicamide (Santen, Osaka, Japan) and potential was recorded via the PowerLab data acquisition system and software Scope (AD Instruments, Dunedin, New Zealand) through a corneal contact lens electrode (Mayo, Aichi, Japan). Peak latencies of oscillatory potentials (OP1, OP2 and OP3) were measured and data was expressed as the sum of OP1 to OP3 (Σ(OP1-OP3)).

Cataracts were evaluated after ERG measurements using a slit lamp (Kowa, Tokyo, Japan). Lens opacity was scored using three grades for each eye as follows: 0 (no opacity; clear lens), 1 (partially clouded lens) and 2 (mature cataract; completely opaque lens). The average score was used as the individual cataract score.

Histology and Immunohistochemistry

At the end of the study, all animals were sacrificed at non-fasted condition by exsanguination under isoflurane anesthesia and necropsy was performed. Kidneys and eyes were fixed in 4% paraformaldehyde and 4% glutaraldehyde / 10% neutral-buffered formalin, respectively. After resection, tissues were paraffin-embedded using standard techniques and thin-sectioned (3–5 μm). The sections were stained with HE for histological evaluation. Intraepidermal nerve fiber density (IENFD) was measured to evaluate small fiber neuropathy (Joint Task Force of the EFNS and the PNS 2010). The skin of hind limbs was dissected and fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned (25 μm). Nerve fibers were immunostained for protein gene product 9.5 (PGP 9.5; rabbit polyclonal, 1:500; UltraClone, Isle of Wight, UK) overnight at 4˚C and Alexa Fluor® mouse anti-rabbit IgG antibody 488 (1:1000, Thermo Fisher Scientific, Waltham, USA) for 30 min at room temperature. Five fields from each section were randomly selected and Z-stack images were obtained using a Nikon A1 confocal laser scanning microscope mounted on an inverted microscope (Nikon, Tokyo, Japan). Nerve fibers with branching inside the epidermis were considered one nerve. IENFDs were expressed as numbers of epidermal nerve fibers per length of the epidermal basement membrane (fibers/mm).

Statistical Analysis

Results are expressed as mean ± or + standard deviation. Statistical analyses of differences between mean values were performed using an F-test, followed by a Student's *t*-test or Aspin-Welch's *t*-test. A Wilcoxon rank-sum test was used for cataract scores. All statistical analyses were performed using the EXSUS statistical analysis

system for biological experiment data (CAC EXICARE, Tokyo, Japan). Differences were accepted as significant at $p < 0.05$.

RESULTS

Hypoglycemic Effect of Phlorizin on SDT Fatty Rats

To confirm the dose of phlorizin for the study, 100 mg/kg of phlorizin was subcutaneously administered once to 18 week-old female SDT fatty rats under non-fasted conditions (Fig.10). Prior to phlorizin treatment, the blood glucose level in the Control group was 370 ± 49 mg/dl. Six hours after dosing, the blood glucose level in the phlorizin treated group decreased to an almost normal level (139 ± 32 mg/dl). The hypoglycemic effect of phlorizin was sustained for 24 hr (224 ± 42 mg/dl); although the effect of phlorizin was weakened at 24 hr, normal blood glucose levels were expected with repeat dosing. Therefore, a single dose of 100 mg/kg phlorizin is sufficient to reduce plasma glucose level for 24 hr in female SDT fatty rats.

Effect of Phlorizin on Biochemical Parameters of SDT fatty rats

Body weights in the Control group were significantly higher than those in the SD group in the experiment. Body weights in phlorizin-treated group rats were heavier than those in the Control group after 12 weeks (Fig. 11A). At the end of the study, food consumption in the Control group was almost twice that in the SD group; however, there were no differences between the Control group and phlorizin-treated group (Fig. 11B). Urinary glucose considerably increased in the Control group, and phlorizin treatment significantly decreased glucose excretion (Fig. 12A).

After 2 weeks, blood glucose levels in the Control group increased to a range

of 424 mg/dl to 663 mg/dl (Fig. 12B). Blood glucose levels were adequately controlled with phlorizin treatment during the experiment and gradually decreased to near normal range (123 mg/dl to 167 mg/dl). Blood glucose levels in the SD group were in the range of 104 mg/dl to 157 mg/dl. HbA1c levels reflected the change of blood glucose level accurately (Fig. 12C). Plasma insulin levels in the Control group were significantly higher than those in the SD group at the beginning of the experiment (6 weeks of age) and gradually decreased to normal level at 20 weeks. Phlorizin treatment delayed insulin decreases and insulin was kept high even at the end of study (Fig. 12D). Plasma TG levels (Fig. 13A), TC levels (Fig. 13B) and FFA levels (Fig. 13C) in the Control group were higher than those in the SD group. TG levels and FFA levels in the phlorizin- treated group were significantly higher and TC levels were lower than those in the Control group only at 7 weeks treatment.

Effect of Phlorizin on Diabetic Nephropathy of SDT fatty rats

After 20 weeks of treatment with phlorizin, urinary parameters were evaluated to assess the effects of phlorizin on diabetic nephropathy. UAE (Fig. 14A) considerably increased in the Control group. Ccr (Fig. 14B) and urinary 8-OHdG (Fig. 14C) also increased significantly. Ccr decreased significantly with phlorizin treatment; however, the effects on UAE and 8-OHdG were limited.

Figs. 14D and 14E show the typical microphotographs of the kidneys in the SD group and the Control group. Slight glomerulosclerosis and tubular regeneration, dilation, Armanni-Ebstein changes and inflammatory cell infiltration in interstitial areas were found in the Control group. Phlorizin treatment prevented Armanni-Ebstein changes (Fig. 14F); however, other renal abnormal findings did not recover with

controlled blood glucose level. Moreover, tubular dilation and inflammatory cell infiltration in the urothelium were frequently observed in phlorizin-treated group.

Effect of Phlorizin on Diabetic Peripheral Neuropathy of SDT fatty rats

At the end of the treatment, sciatic MNCV and SNCV were measured under anesthesia. Both MNCV and SNCV were significantly delayed in the Control group compared with the SD group. The MNCV in the Control group decreased to 82.0% of that in the SD group and SNCV decreased to 82.4% (Figs. 15A and 15B). These functional impairments in nerves were corrected to 95.6% and 98.3% of those in the SD group, respectively, with 23 weeks of phlorizin administration.

To evaluate peripheral nerve density, skin biopsies with quantification of IENFD were performed. IENFD from the skin of the hind paw foot in the Control group decreased significantly after 23 weeks (SD group; 27.1 ± 2.3 fibers/mm, Control group; 14.8 ± 3.1 fibers/mm). 23 weeks of phlorizin treatment prevented the decrease of nerve fibers (23.6 ± 3.2 fibers/mm) (Figs. 16A-16D).

Effect of Phlorizin on Diabetic Retinopathy and Cataracts of SDT fatty rats

At Week 23, the Control group showed prolongations of peak latencies of oscillatory potential in ERGs compared with the SD group. There was a significant prolongation of peak latency for each individual oscillatory potential (OP1-OP3), as well as that of the summed potential Σ(OP1-OP3). Prolongation of these peak latencies decreased significantly with 23 weeks of phlorizin administration (Fig. 17A). Histopathologically, retinal folding was observed in the Control group as previously reported (Kemmochi *et al.* 2013; Ohta *et al.* 2014b). Retinal abnormalities were

completely prevented with phlorizin (Figs. 17C-17E).

Cataracts progressed in SDT fatty rats from 8 weeks of age. At Week 23, all SDT fatty rats in the Control group showed mature cataracts via macroscopic observation. Cataracts in phlorizin-treated group did not progress even at the end of experiments (Figs. 17B and 17F-17H).

DISCUSSION

The SDT fatty rat has been developed as a new type 2 diabetes model with rapidly progressing diabetic microvascular complications (Kemmochi *et al*. 2013; Masuyama *et al*. 2005; Matsui *et al*. 2008b). SDT fatty rats develop diabetes from 5 weeks of age, and the incidence of diabetes in both male and female SDT fatty rats is 100%. Previously, we evaluated some hypoglycemic drugs such as pioglitazone, metformin and DPP IV inhibitor, on male SDT fatty rats (Fukuda *et al*. 2011; Yamaguchi *et al*. 2012). Despite that the female SDT fatty rats also show severe diabetes and its complications, only few experiments have been reported (Yamaguchi *et al*. 2012). Therefore, in the present study, we used female SDT fatty rats to investigate the characteristics of diabetic complications in this animal model by controlling blood glucose level with phlorizin. We have confirmed that phlorizin treatment sufficiently reduce blood glucose level in male SDT rats (unpublished data). Phlorizin is a non-selective SGLT inhibitor and recently-launched SGLT2 inhibitors are treated as anti-hyperglycemic drugs with a novel mechanism of action. Although it is difficult to distinguish the effect on SGLT1 in gut from that on SGLT2 in kidney, the changes in body weight, blood glucose levels and HbA1c suggest the usefulness of SGLT inhibitor phlorizin in the treatment of hyperglycemia. By using phlorizin, primarily

hypoglycemic effects are expected without affecting other biochemical parameters. Pharmacological effects of phlorizin on diabetic animal models were reported previously. In concurrence with our result, phlorizin treatment prevented hyperglycemia and preserved insulin mRNA levels but failed to prevent hypertriglyceridemia in ZDF rats (Harmon *et al*. 2001).

Body weights of SDT fatty rats are obviously heavier than normal SD rats because of hyperphagia due to the lack of leptin signaling (Masuyama *et al*. 2005; Matsui *et al*. 2008b). With the progress of hyperglycemia, body weight gain gradually slowed down. Repeated treatment with phlorizin completely prevented the increase of plasma glucose levels during the experiment. Improvement of hyperglycemia with phlorizin is considered to influence general condition, leading to further increases in body weight.

For the kidneys of SDT fatty rats, we previously reported histopathological changes in glomeruli (glomerulosclerosis, increased mesangial matrix and glomerular hypertrophy, and nodular lesions) and renal tubules (glycogen deposition (Armanni-Ebstein lesions), fibrosis, inflammatory cell filtration and tubular dilation) (Ishii *et al*. 2010b; Kemmochi *et al*. 2013; Matsui *et al*. 2008b). In the present study, SDT fatty rats showed significant increases in urinary glucose, UAE, and Ccr. The primary effect of phlorizin is inducing glucosuria; however, urinary glucose levels in the phlorizin treated group were lower than the vehicle treated group. Lower glucosuria may reflect reduced plasma glucose levels in phlorizin treated SDT fatty rats at these time points. These data are consistent with previous report that hyperglycemia was decreased by phlorizin in STZ-induced diabetes rats but significant diuresis and glucosuria remain (Osorio *et al*. 2012). Although creatinine clearance decreased

significantly, UAE and 8-OHdG were partially decreased with phlorizin treatment; these parameters did not recover completely by controlling only blood glucose level. In addition to hyperglycemia, abnormal lipid metabolism has been considered an important factor in the pathogenesis of diabetic nephropathy (Guijarro *et al*. 1995; Hsu *et al*. 2000; Sun *et al*. 2002). Since phlorizin treatment failed to reduce blood TG and TC levels, uncontrolled dyslipidemia may affect diabetic nephropathy in SDT fatty rats. In the report using STZ rats, phlorizin prevented proteinuria, hyperfiltration and whole kidney hypertrophy, but not glomerular hypertrophy. Therefore, some part of renal impairment in STZ rats is uncontrollable with decreasing blood glucose level alone (Malatiali *et al*. 2008). On the other hand, some histopathological findings were observed in the phlorizin-treated group. Increased UV and susceptibility to urinary tract infection by inhibiting SGLT2 in proximal renal tubules may lead to tubular dilation and inflammatory cell infiltration in the urothelium of phlorizin treated SDT fatty rats.

We reported that caudal MNCV in both male and female SDT fatty rats was delayed at 24 weeks of age (Katsuda *et al*. 2014; Yamaguchi *et al*. 2012). Histopathologically, at 40 weeks of age, significant decreases in sural nerve fiber number due to atrophy were observed in male SDT fatty rats (Yamaguchi *et al*. 2012). In the present study, we evaluated IENFD in SDT fatty rats as a hallmark of small fiber neuropathy. IENFD is considered a marker of diabetic peripheral neuropathy that reduces from early stage diabetes (Shun *et al*. 2004). Similar to other diabetic animal models (Brussee *et al*. 2008; Himeno *et al*. 2011), SDT fatty rats showed significantly decreased IENFD. Significant histopathological and functional preservation of peripheral nerves were achieved with phlorizin. As is the case with diabetic nephropathy, effects of dyslipidemia and hypertension on diabetic peripheral neuropathy have been

pointed out (Goncalves *et al*. 2009; Obrosova *et al*. 2007). In contrast to diabetic nephropathy, these factors have insignificant effects on diabetic peripheral neuropathy in SDT fatty rats because controlling only blood glucose level prevented histopathological and functional nerve impairments.

As a marker of diabetic retinopathy, retinal function was evaluated using ERGs. Previously, we reported the ERG results in SDT fatty rats; prolongation of OPs was observed in both males (16 weeks of age) and females (22 weeks of age) (Katsuda *et al*. 2014; Matsui *et al*. 2008b). Delayed OPs were also observed in the present study and phlorizin treatment prevented retinal dysfunction. Histopathological changes in the lens, such as hyperplasia of the epithelium, vacuolation of fibers, and formation of Morgagnian globules, coincide with previous reports (Ishii *et al*. 2010b; Matsui *et al*. 2008b). Furthermore, retinal lesions, such as folding and thickening, which are found in aged SDT fatty rats (Katsuda *et al*. 2014; Kemmochi *et al*. 2013), were also observed. These histopathological abnormalities were not observed in the eyes of SDT fatty rats treated with phlorizin. Although dyslipidemia is a key factor of diabetic retinopathy (ACCORD Study Group *et al*. 2010), our findings suggest that hyperglycemia is the major cause of diabetic retinopathy and other ocular changes in SDT fatty rats.

In conclusion, diabetic complications in SDT fatty rats are caused by sustained severe hyperglycemia. Notably, other factors than hyperglycemia, such as hyperlipidemia and hypertension may be involved in diabetic nephropathy in SDT fatty rats. In addition, we reported that enhanced vascular endothelial growth factor (VEGF) signaling also contributes to microvascular dysfunction in SDT rats recently (Mukai *et al*. 2014). Clarifying the mechanism of diabetic complications further enhances the prospects for the usefulness of this animal model in developing new drugs and therapies

for diabetic microvascular complications.

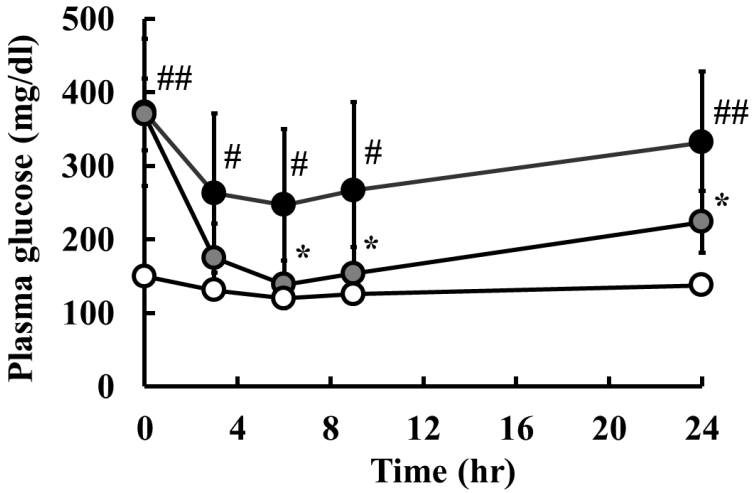

Figure 10 Hypoglycemic effect of phlorizin in SDT fatty rats. A single dose of phlorizin clearly decreased blood glucose levels. Six hours after administration, blood glucose reached normal levels. At 24 hours, the effect of phlorizin partially remained. White circles; SD group, black circles; Control group, gray circles; phlorizin treated group. Each value represents the mean ± standard deviation (n=4). *$p < 0.05$; significantly different from the Control group, #$p < 0.05$, ##$p < 0.01$; significantly different from the SD group.

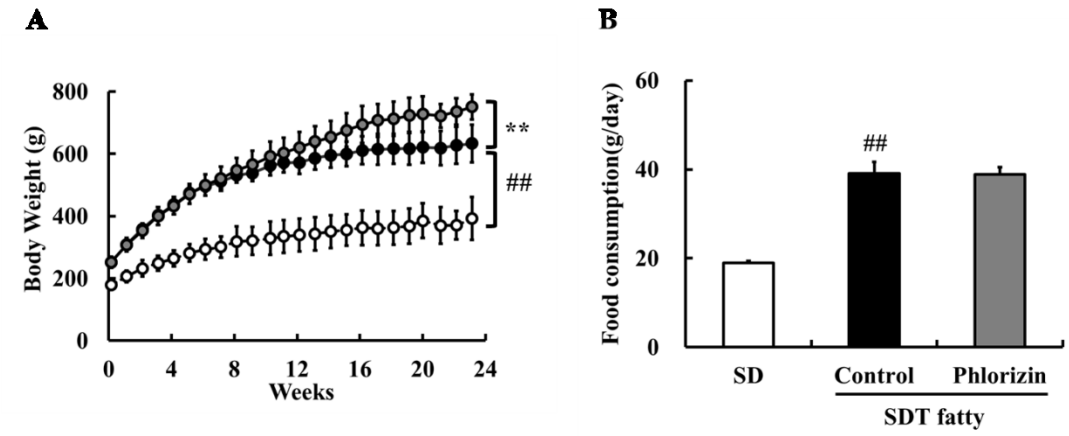

Figure 11 Effect of phlorizin on biochemical parameters of SDT fatty rats. Effects of phlorizin on body weight (A) and food consumption (B) (at the end of experiment; 29 weeks of age). White circles; SD group, black circles; Control group, gray circles; phlorizin treated group. Each value represents the mean ± or + standard deviation (n=8). **$p < 0.01$; significantly different from the Control group, ##$p < 0.01$; significantly different from the SD group.

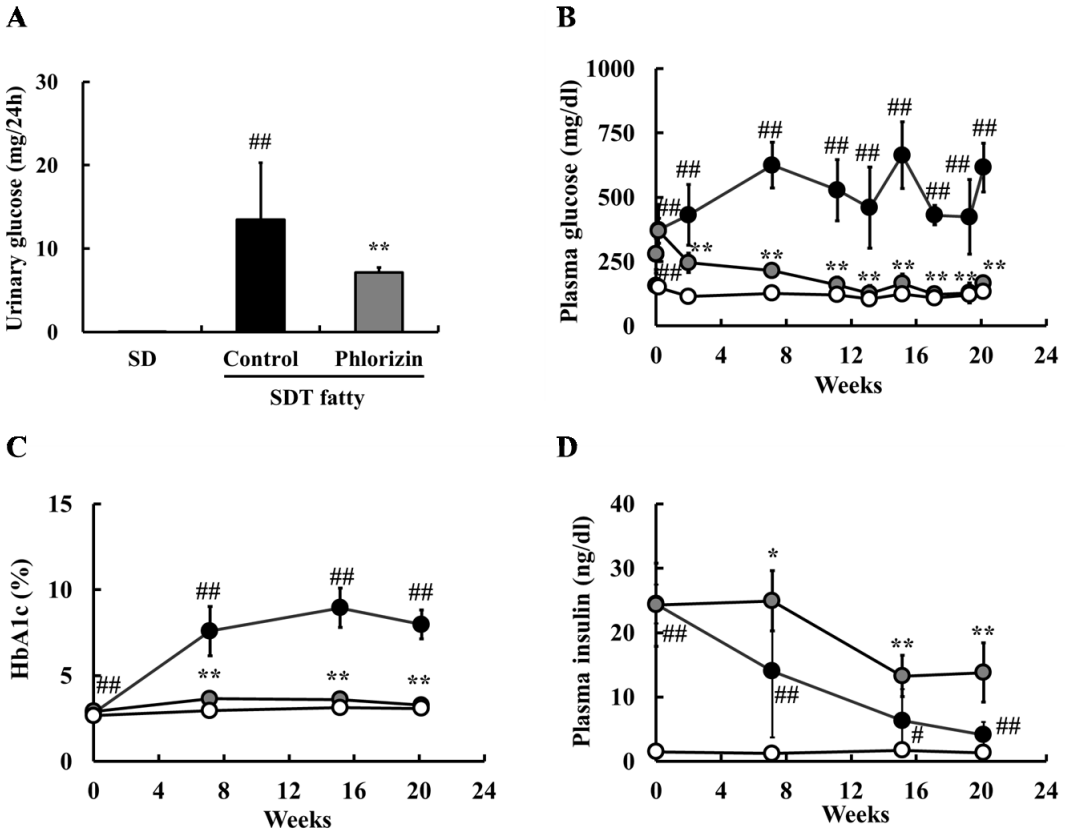

Figure 12 Effect of phlorizin on biochemical parameters of SDT fatty rats. Effects of phlorizin on urinary glucose (A), plasma glucose levels (B), hemoglobin A1c (HbA1c) levels (C) and plasma insulin levels (D). Phlorizin treatment improved hyperglycemia and delayed insulin level deceases. White circles; SD group, black circles; Control group, gray circles; phlorizin treated group. Each value represents the mean + or ± standard deviation (n=8). *$p < 0.05$, **$p < 0.01$; significantly different from the Control group, #$p < 0.05$, ##$p < 0.01$; significantly different from the SD group.

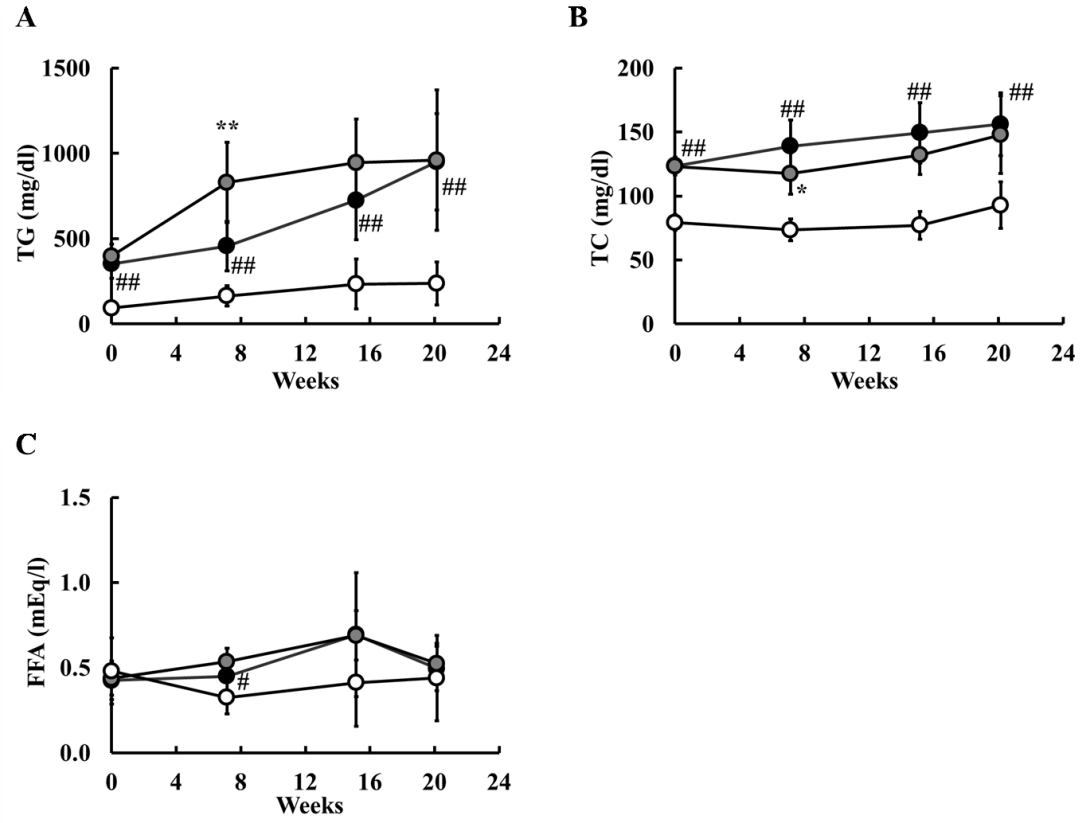

Figure 13 Effect of phlorizin on biochemical parameters of SDT fatty rats. Effects of phlorizin on plasma triglyceride (TG) levels (A), plasma total cholesterol (TC) levels (B) and plasma free fatty acid (FFA) levels (C). Hyperlipidemia was not clearly prevented with phlorizin. White circles; SD group, black circles; Control group, gray circles; phlorizin treated group. Each value represents the mean ± standard deviation (n=8). *$p < 0.05$, **$p < 0.01$; significantly different from the Control group, #$p < 0.05$, ##$p < 0.01$; significantly different from the SD group.

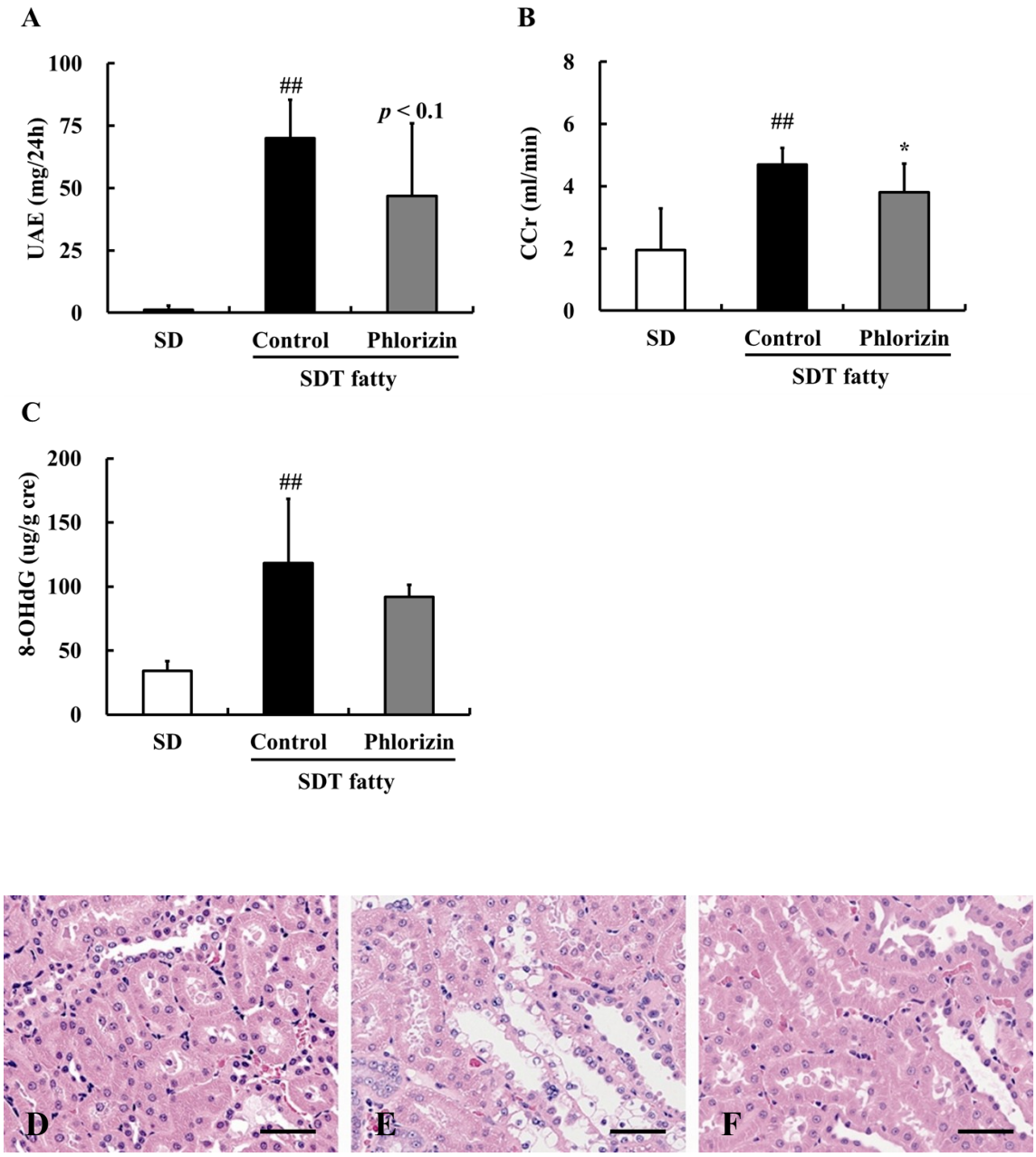

Figure 14 Effect of phlorizin on diabetic nephropathy of SDT fatty rats. Urinary albumin excretion (UAE) (A), creatinine clearance (Ccr) (B) and urinary 8-Hydroxydeoxyguanosine (8-OHdG) (C) increased in the Control group at after 20 weeks. Increases in all these renal parameters were partially prevented with phlorizin treatment. Typical microphotographs of the kidneys in the SD group (D) and the Control

group (E). Tubular dilation and Armanni-Ebstein changes were found in the Control group. Phlorizin treatment completely prevented these tubular abnormalities (F). Bars = 50 μm. Each value represents the mean + standard deviation (n=8). *$p < 0.05$; significantly different from the Control group, ##$p < 0.01$; significantly different from the SD group.

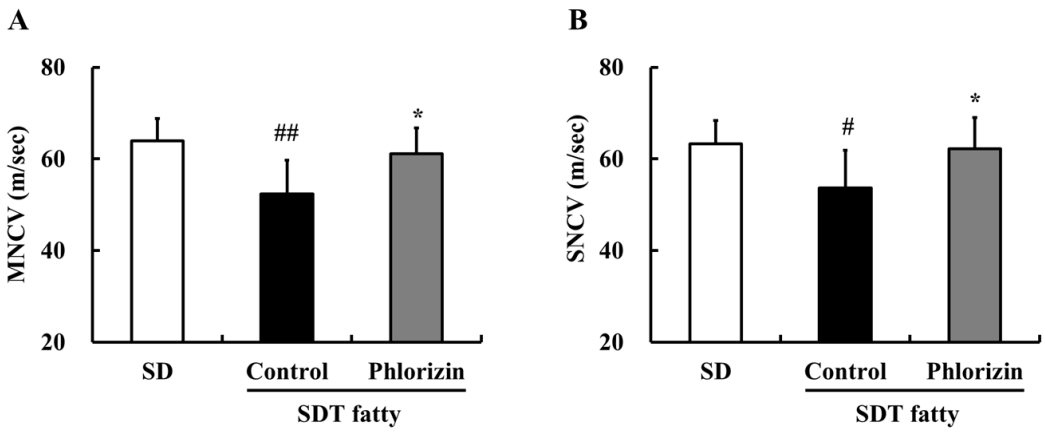

Figure 15 Effect of phlorizin on diabetic peripheral neuropathy of SDT fatty rats. The effects of phlorizin on peripheral nerve dysfunction (sciatic motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV)) in female SDT fatty rats were evaluated. Impairments of MNCV (A) and SNCV (B) improved significantly with phlorizin treatment compared with the Control group. Each value represents the mean + standard deviation (n=6-8). *$p < 0.05$; significantly different from the Control group, #$p < 0.05$, ##$p < 0.01$; significantly different from the SD group.

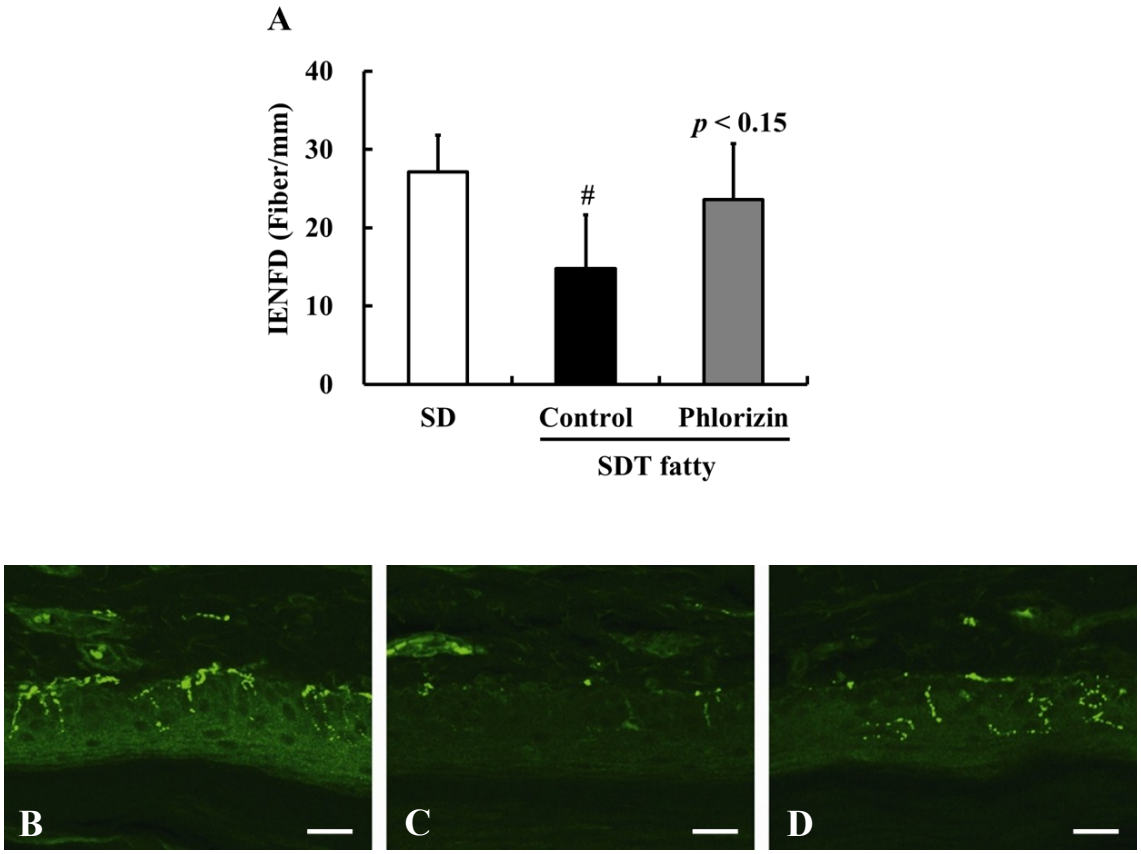

Figure 16 Effect of phlorizin on diabetic peripheral neuropathy of SDT fatty rats. The effects of phlorizin on intraepidermal nerve fiber density (IENFD) in female SDT fatty rats were evaluated. Reductions in IENFD were prevented with phlorizin (A). Typical confocal microscopic pictures of anti-PGP9.5 immunostained hind paw skin from the SD group (B), Control group (C), and phlorizin-treated group (D). Bars = 20 μm. Each value represents the mean + standard deviation (n=6-8). #$p < 0.05$; significantly different from the SD group.

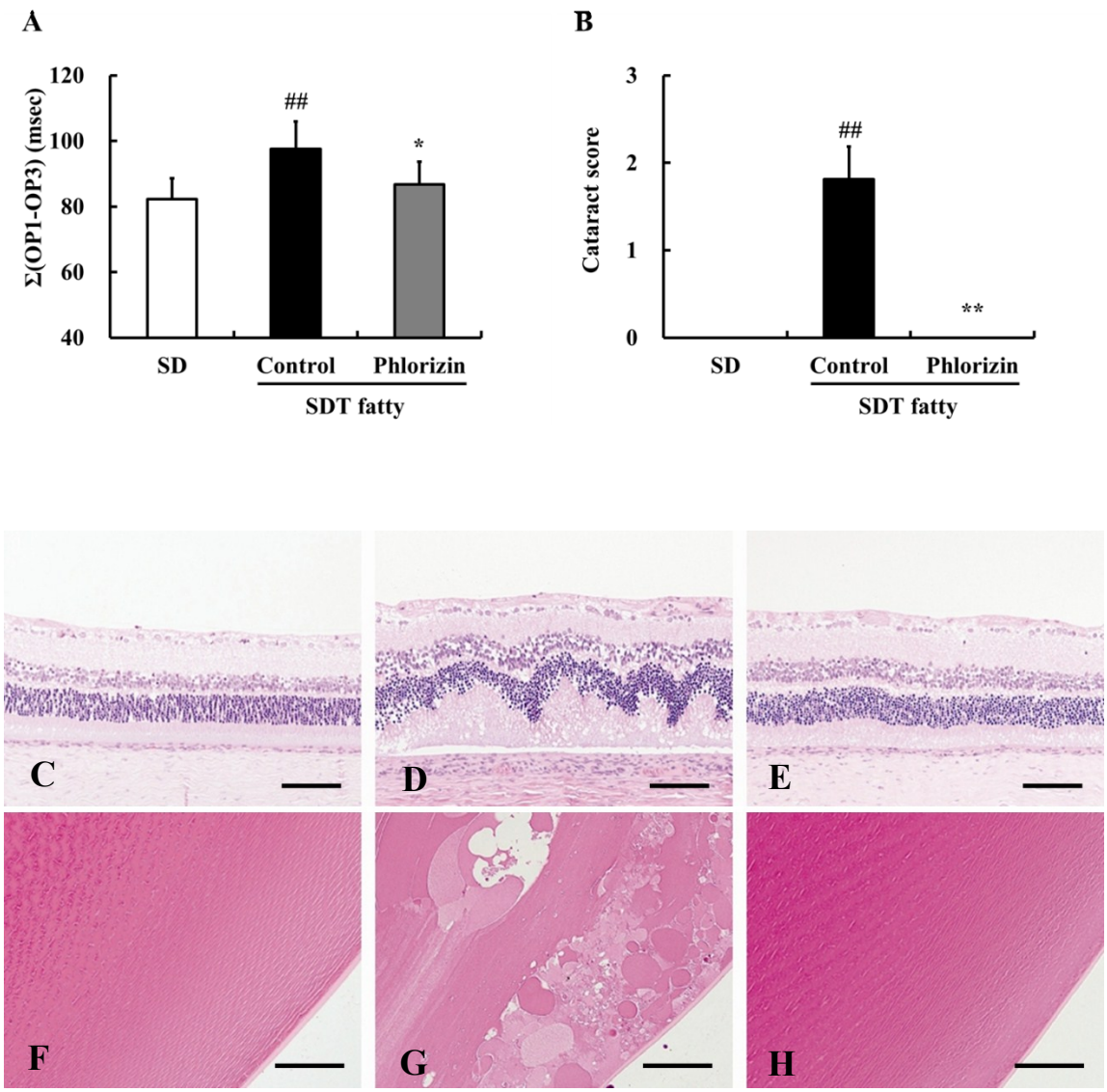

Figure 17 Effect of phlorizin on diabetic retinopathy and cataracts of SDT fatty rats. Delayed oscillatory potentials in electroretinogram (ERG) were observed in the Control group (A). Cataracts also progressed in the Control group (B). These eye disorders were prevented with phlorizin treatment. Compared to the SD group (C), retinal folding and thickening were observed in the Control group (D) and were improved with phlorizin (E). Bars = 100 μm. SD group showed clear lens (F), but mature cataracts were found in the Control group (G). Cataracts did not progress in phlorizin treated group (H). Bars =

50 μm. Each value represents the mean + standard deviation (n=8). *$p < 0.05$, **$p < 0.01$; significantly different from the Control group, ##$p < 0.01$; significantly different from the SD group.

Chapter 5

Contribution of hypertension to diabetic nephropathy in obese type 2 diabetic SDT fatty rats: effects of angiotensin receptor blocker losartan

INTRODUCTION

The pathology of renal disease in patients with type 2 diabetes is complex and, in addition to hypertension, factors such as hyperglycemia and dyslipidemia contribute to the development of renal disease in a complex manner (Calcutt *et al*. 2009).

The kidneys control blood pressure and an impairment or failure of renal function causes hypertension. The increase in cardiac output and peripheral vascular resistance also leads to hypertension. The activation of the sympathetic nervous system, decline in glomerular filtration rate, increase in circulating blood volume due to failure of sodium excretion, and elevation in heart rate and cardiac all contractility result in an increase in cardiac output. On the other hand, in addition to the activation of the sympathetic nervous system, vasoconstriction due to the renin-angiotensin system affects the increase in peripheral vascular resistance.

At present, there are several antihypertensive drugs, such as beta-blockers, calcium channel blockers and renin-angiotensin blockers. To treat diabetic nephropathy, renin-angiotensin blockers are used as first-line treatment. Angiotensin II receptor blockers, such as losartan and irbesartan, are widely prescribed domestically and abroad. There are two subtypes of angiotensin receptors, angiotensin II type 1 receptors and angiotensin II type 2 receptors. Angiotensin II induces the contraction of vascular smooth muscle cells and the release of aldosterone via the angiotensin II type 1 receptor. In 1152 type 2 diabetic patients with diabetic nephropathy who took part in a

multicenter collaborative trial, genetic polymorphisms in the angiotensin-converting enzyme and the association of angiotensin II type 1 receptor with the progression and deterioration of diabetic nephropathy were reported in genetic analyses of the relationship between the renin-angiotensin system and diabetic nephropathy with type 2 diabetes (Tomino *et al.* 1999).

In this study, renal parameters and pathohistological changes in kidneys of unilaterally nephrectomized SDT fatty rats were evaluated to understand the effects of hypertension on the development and progression of diabetic nephropathy using the antihypertensive drug losartan. The conduct of this study is detailed in Chapter 2.

MATERIALS AND METHODS

Animals and Chemicals

Male SDT fatty rats from Japan Tobacco colonies were used in this study. At eight weeks of age, SDT fatty rats were divided into 3 groups: a losartan-treated group, 1/2 nephrectomy group (1/2 Nx group) and sham operation group (Control group) (n=8-10). Animals at 8 weeks of age underwent 1/2 Nx or sham surgery under anesthesia. In the 1/2 Nx group and the losartan-treated group, a small lumbar incision was made, and the left kidney was removed. In the Control group, the left kidney was exposed and gently manipulated but left intact. Losartan (LKT laboratories, MN, USA) mixed into the diet at a concentration of 0.2% was administered for 10 weeks starting from 8 weeks of age.

Animals were housed in suspended bracket cages and given a standard laboratory diet (CRF-1; Oriental Yeast, Tokyo, Japan) and water in a room with controlled temperature, humidity, and lighting (temperature 23 ± 3°C, humidity 55 ±

15%, 12 h lighting cycle). This experiment was conducted in compliance with the Guidelines for Animal Experimentation established for our biological/pharmacological laboratories.

Biophysiological Parameters

Body weight and biochemical parameters were assessed from Week 2 to 10, every 2 weeks. Blood samples were collected from the tail vein of non-fasted rats. Serum glucose, TG, and TC were measured as biochemical parameters using commercial kits (F. Hoffmann-La Roche, Basel, Switzerland) and an automatic analyzer (Hitachi, Tokyo, Japan).

Systolic Blood Pressure and Heart Rate

SBP and heart rate in a conscious non-fasted state were measured at Week 4 and 8 by the indirect tail cuff method using a Softron BP-98A indirect blood pressure meter (Softron, Tokyo, Japan). Blood pressure and heart rate were measured between 13:00 and 16:00.

Renal Parameters

UV, urinary albumin, BUN, and Ccr were evaluated as renal parameters. Blood samples were collected from the tail vein of non-fasted rats. Urine samples were collected by placing the animals in metabolic cages with water for 6 h. Urinary albumin was measured using a rat-albumin EIA kit (Panapharm Laboratories, Kumamoto, Japan). Urinary creatinine, serum creatinine, and BUN were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi, Tokyo,

Japan). Ccr was calculated by dividing the urinary excretion of creatinine at 6 h by serum creatinine level and body weight.

Histopathological Examination

Necropsy was performed at the end of the experiment and kidneys were collected from all animals. Kidneys were weighed and fixed with 4% paraformaldehyde. After resection, the tissues were paraffin-embedded using standard techniques and thin-sectioned (3–5 μm). Sections were stained with HE and PAS. Eight mice in the 1/2 Nx group, nine mice in the losartan-treated group and five mice in the Control group were examined histopathologically in a blinded manner.

Statistical Analysis

Results were expressed as means ± or + standard deviation. Statistical analyses of differences between mean values in the 1/2 Nx group and the losartan-treated group or the Control group were performed using the F-test, followed by Student's *t*-test or Aspin-Welch's *t*-test. Differences were defined as significant when $p < 0.05$.

RESULTS

Body Weight and Biochemical Parameters

Body weight was comparable among groups during the experimental period (Control group, 521.2 ± 40.3 g; 1/2 Nx group, 479.7 ± 116.2 g; losartan-treated group, 493.9 ± 87.8 g, at 10 weeks of treatment).

Plasma glucose levels in the losartan-treated group were similar to those in the Control group and the 1/2 Nx group during the experimental period (Control group,

799.9 ± 76.9 mg/dl; 1/2 Nx group, 751.0 ± 63 mg/dl; losartan-treated group, 820.7 ± 62 mg/dl, at 10 weeks of treatment) (Fig. 18A). Serum TG levels in the 1/2 Nx group at Week 10 were significantly higher than those in the Control group. Treatment with losartan tended to decrease serum TG levels; however, the effects on serum TG levels were not significant (Control group, 309.6 ± 67.4 mg/dl; 1/2 Nx group, 501.9 ± 211.4 mg/dl; losartan-treated group, 388.9 ± 257.4 mg/dl, at 10 weeks of treatment) (Fig. 18B). Serum TC levels tended to increase in the 1/2 Nx group during the experimental period. Treatment with losartan tended to decrease serum TC levels; however, the effects on serum TC levels were not significant (Control group, 120.8 ± 18.7 mg/dl; 1/2 Nx group, 154.4 ± 51.7 mg/dl; losartan-treated group, 131.8 ± 31.2 mg/dl, at 10 weeks of treatment) (Fig. 18C).

Systolic Blood Pressure and Heart Rate

SBP at Week 4 and 8 in the 1/2 Nx group were significantly elevated compared with those in the Control group. SBP decreased significantly with losartan treatment (Control group, 125.3 ± 14.3 mmHg; 1/2 Nx group, 154.0 ± 22.1 mmHg; losartan-treated group, 132.1 ± 16.4 mmHg, at 8 weeks of treatment) (Fig. 19A). For heart rate, there were no differences among groups (Control group, 287.5 ± 29.0 beat/min; 1/2 Nx group, 301.6 ± 41.9 beat/min; losartan-treated group, 308.2 ± 40.1 beat/min, at 8 weeks of treatment) (Fig. 19B).

Renal Parameters

Renal parameters, such as UAE, serum BUN and Ccr, were evaluated to assess the effects of losartan on renal function from Week 2 to 10 of the treatment period,

every 2 weeks. UAE in the 1/2 Nx group increased from Week 6 compared with those in the Control group. However, treatment with losartan failed to suppress the increase in UAE (Control group, 3458.4 ± 681.1 μg/mg creatinine; 1/2 Nx group, 7324.2 ± 1367.8 μg/mg creatinine; losartan-treated group, 6613.7 ± 2568.2 μg/mg creatinine, at 10 weeks of treatment) (Fig. 20A). Serum BUN levels in the 1/2 Nx group were significantly higher than those in the Control group during the experimental period. However, treatment with losartan failed to suppress the increase in serum BUN levels (Control group, 27.6 ± 3.3 mg/dl; 1/2 Nx group, 35.3 ± 2.8 mg/dl; losartan-treated group, 37.3 ± 3.5 mg/dl, at 10 weeks of treatment) (Fig. 20B). Ccr in the 1/2Nx group tended to decrease compared with that in the Control group at 10 weeks of treatment. However, treatment with losartan did not suppress the decrease in Ccr (Control group, 0.31 ± 0.06 ml/h*g; 1/2 Nx group, 0.23 ± 0.12 ml/h*g; losartan-treated group, 0.27 ± 0.09 ml/h*g, at 10 weeks of treatment).

Histopathological Examination of the Kidney

Kidney weights in the 1/2 Nx group were higher than those in the Control group. Treatment with losartan did not affect kidney weights (Control group, 2.3 ± 0.2 g; 1/2 Nx group, 3.9 ± 0.3 g; losartan-treated group, 3.6 ± 0.4 g, at 10 weeks of treatment) (Fig. 20C).

The results of histopathological examinations of the kidney at Week 10 are shown in Table 3. The following findings in the glomerulus, tubule, and interstitium were observed in both the Control group and the 1/2 Nx group. Glomerulosclerosis was characterized by an increase in the size of the glomerulus and diffuse thickening of the glomerulocapillary wall, as well as mesangial expansion, showing partly segmental

fibrosis in severe cases. Tubular lesions included tubular regeneration, dilatation, hyaline casts, and interstitial lesions, including fibrosis and inflammatory cell infiltration. The histological features of the kidney were not different between the 1/2 Nx group and the Control group; however, the pathological degree observed in the 1/2 Nx group was more progressive. Treatment with losartan did not improve histopathological changes in the kidney compared with those in the 1/2 Nx group.

DISCUSSION

In this study, renal parameters and histopathological changes in kidneys of unilaterally nephrectomized SDT fatty rats were evaluated to understand the effects of hypertension on the development and progression of diabetic nephropathy using the antihypertensive drug losartan.

Unilaterally nephrectomized SDT fatty rats showed elevated blood pressure and increases in UAE. Treatment with losartan decreased blood pressure to levels similar to those observed in the Control group (Fig. 19A); however, UAE did not improve with controlled blood pressure. Angiotensin II type 1 receptor blockers are known to decrease urinary protein excretion in type 2 diabetic patients (Lewis *et al*. 2001; Brenner *et al*. 2001; Parving *et al.* 2001; Lacourciere *et al*. 2000) and diabetic animal models (Uehara *et al*. 1999; Inada *et al.* 2000). Angiotensin II type 1 receptor blockers, such as E4177 and TA6069, decreased blood pressure to levels similar to those in Long-Evans Tokushima Otsuka (LETO) rats and suppressed urinary protein excretion in an obese type 2 diabetic animal model, OLETF rats (Inada *et al*. 2000). The angiotensin II type 1 receptor blocker, olmesartan, also decreased blood pressure and urinary protein excretion in an obese type 2 diabetic animal model, ZDF rats, and these

effects are correlated with a decrease in blood pressure (Mizuno *et al.* 2002).

In contrast, the control of blood pressure did not improve renal function in unilaterally nephrectomized SDT fatty rats. These results suggest that factors contributing to the development and progression of diabetic nephropathy in SDT fatty rats are different from those in the aforementioned animal models. Hyperglycemia and hypertension are major risk factors associated with diabetic nephropathy. In addition to these factors, there is growing evidence that dyslipidemia also contributes to renal disease progression. Dyslipidemia reportedly accelerates the rate of progression of renal disease in a variety of animal models (Abrass 2004). A high-fat diet induces macrophage infiltration and foam cell formation in rats, leading to glomerulosclerosis (Hattori *et al.* 1999). Hyperlipidemia and hyperglycemia also act synergistically to induce renal injury in low density lipoprotein (LDL) receptor-deficient mice (Spencer *et al.* 2004). These findings indicate that lipids can exacerbate renal disease. SDT fatty rats show lipid abnormalities starting from a younger age. However, treatment with losartan significantly failed to reduce blood TG and TC levels. Uncontrolled dyslipidemia may affect renal disease in SDT fatty rats.

Pharmacotherapeutics for diabetic nephropathy mainly focus on the improvement of progressive risk factors, such as diabetes and hypertension. Since most of the patients with diabetic nephropathy have hypertension, pharmacotherapeutics in the clinic are basically antihypertensive drugs, such as renin-angiotensin blockers. Diabetic nephropathy is classified into five different disease stages based on urinary albumin level and estimated glomerular filtration rate (eGFR): glomerular hyperfiltration (stage 1), incipient nephropathy (stage 2), microalbuminuria (stage 3), overt proteinuria (stage 4) and ESRD (stage 5) (Lizicarova *et al*. 2014; Mogensen *et al*.

1983). Angiotensin II type 1 receptor blockers are effective in patients with stage 2 CKD and are not effective in patients with stage 3 or higher CKD. Therefore, the development of novel therapies and therapeutic drugs is required for patients with stage 3 or higher CKD.

In conclusion, the present study revealed that the control of blood pressure failed to improve nephropathy in unilaterally nephrectomized SDT fatty rats and worsening factors of diabetic nephropathy in SDT fatty rats were different from those in other diabetic animal models. These multiple factors may contribute to the development and progression of diabetic nephropathy in SDT fatty rats in a complex manner. Further investigation is necessary for the development of new drugs with novel mechanisms of action for patients that do not experience apparent effects on renal function using pre-existing drugs.

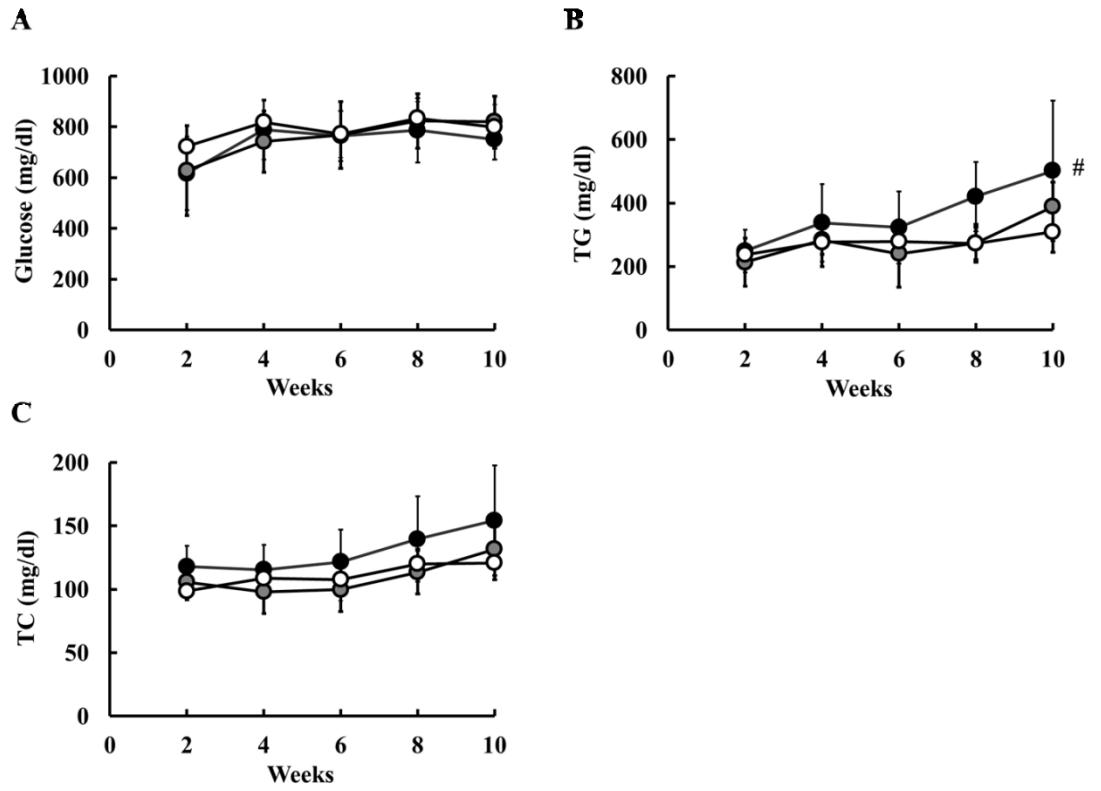

Figure 18 Effect of losartan on serum glucose (A), triglyceride (TG) (B), and total cholesterol (TC) (C) levels in unilateral nephrectomized SDT fatty rats. White circles; Control group, black circles; 1/2 Nx group, gray circles; losartan-treated group. Data represent means ± standard deviation (n=8-10). #p <0.05; significantly different from the Control group.

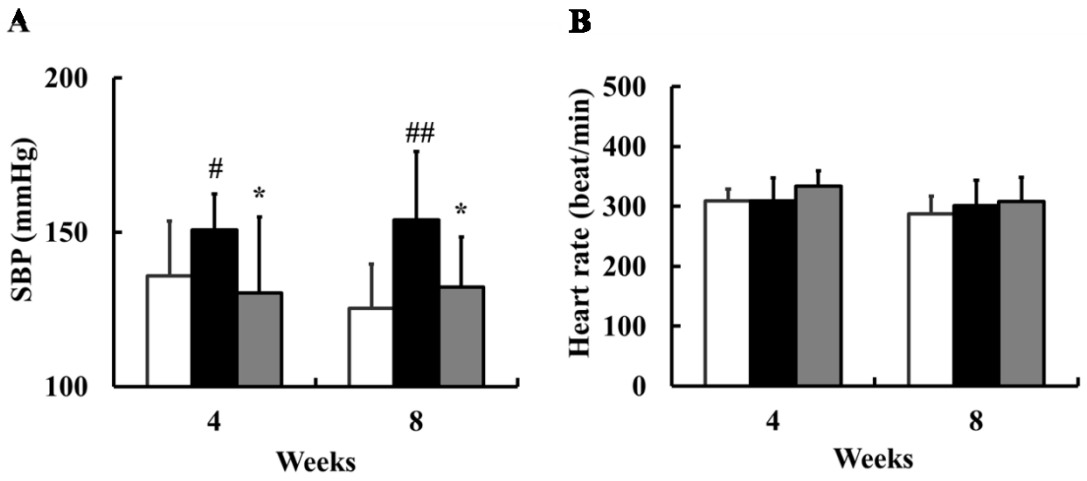

Figure 19 Effect of losartan on systolic blood pressure (SBP) (A) and heart rate (B) in unilateral nephrectomized SDT fatty rats. White bars; Control group, black bars; 1/2 Nx group, gray bars; losartan-treated group. Data represent means + standard deviation (n=8-10). **p* <0.05; significantly different from the 1/2 Nx group. #*p* <0.05, ##*p* <0.01; significantly different from the Control group.

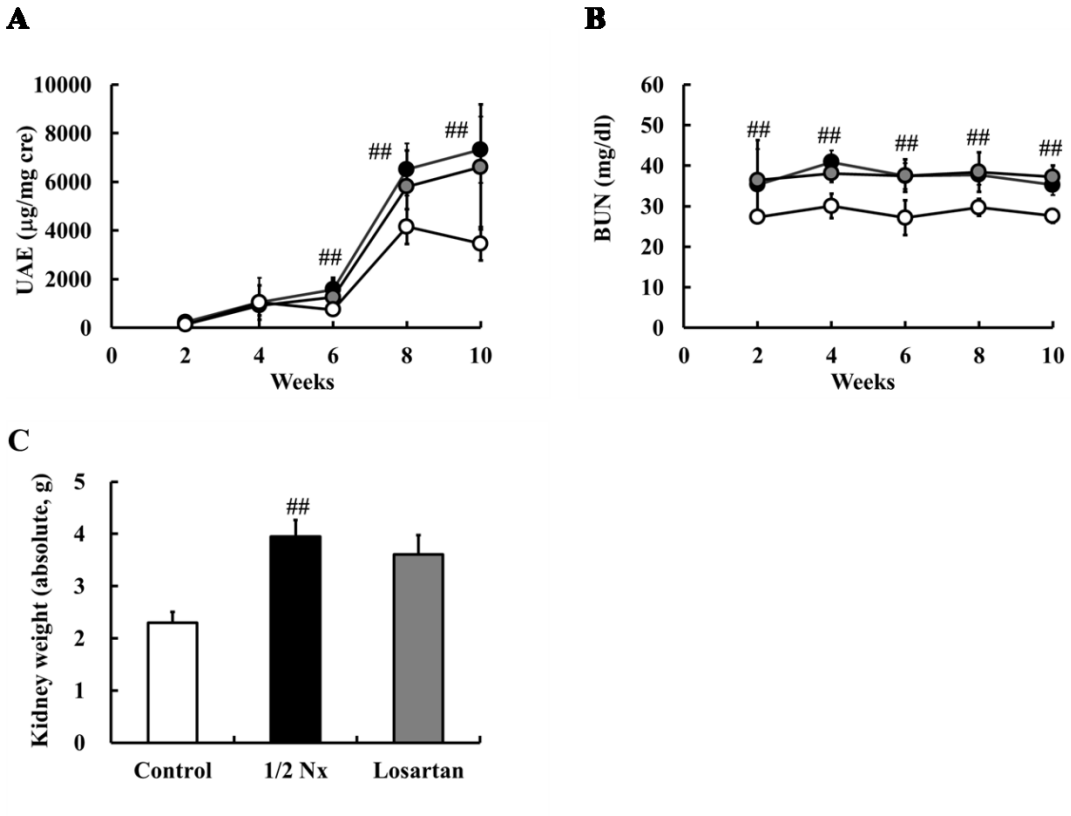

Figure 20 Effect of losartan on urinary albumin excretion (UAE) (A), blood urea nitrogen (BUN) (B), and kidney weight (C) in unilateral nephrectomized SDT fatty rats. White circles; Control group, black circles; 1/2 Nx group, gray circles; losartan-treated group. Data represent means ± or + standard deviation (n=8-10). ##p <0.01; significantly different from the Control group.

Table 3 Histopathological findings of kidney in the 1/2 Nx group, the Losartan-treated group, and the Control group

Group	1/2 Nx								Losartan									Control				
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Findings																						
Glomerulus																						
Glomerulosclerosis	+	+	2+	2+	2+	+	+	2+	+	2+	+	±	2+	2+	+	+	2+	-	±	±	+	-
Tubule																						
Regeneration	+	2+	2+	+	2+	+	2+	2+	+	+	2+	+	2+	2+	2+	+	2+	±	-	+	+	±
Dilatation	+	2+	2+	+	+	2+	2+	2+	+	+	2+	+	2+	2+	+	+	2+	±	+	±	±	±
Hyaline cast	+	+	+	+	+	+	+	+	±	+	+	+	2+	+	2+	+	+	±	±	±	-	±
Deposit, hyaline droplet	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Armanni-Ebstein change	+	+	+	±	+	+	+	+	+	+	±	+	+	+	+	±	+	+	+	+	+	+
Mineralization	-	+	-	-	+	-	-	-	-	-	+	-	±	-	±	-	-	±	-	-	±	±
Interstitial																						
Fibrosis, interstitial	±	-	+	±	±	+	+	±	+	+	+	±	+	+	+	±	±	-	-	-	±	-
Infiltration, inflammatory cell, interstitial	2+	+	2+	+	2+	2+	+	+	+	2+	+	2+	+	+	2+	+	2+	+	+	±	+	-

–: Negative, ±:Very slight, +: Slight, 2+: Moderate, 3+: Severe

Chapter 6

General Discussion

In recent years, the number of patients with diabetic nephropathy has been increasing on a global scale (Hall *et al.* 2003; Ritz & Stefanski 1996). Due to the complexity of the pathology of diabetic nephropathy, factors that are deeply involved in the reduction of renal function in diabetic nephropathy are not clear. Therefore, effective therapeutic approaches for the treatment of diabetic nephropathy are currently lacking. In these circumstances, kidney pathological analysis studies using animal models are extremely important for the development of novel therapies for diabetic nephropathy. The SDT fatty rat is a novel obese diabetic animal model and is considered to exhibit human-like characteristics of diabetic nephropathy (Matsui *et al.* 2008b; Katsuda *et al*. 2014). However, studies that focus on the pathogenesis and mechanism of the development and progression of diabetic nephropathy in SDT fatty rats have not been performed. To clarify the pathophysiological characteristics of diabetic nephropathy in SDT fatty rats, the effects of unilateral nephrectomy or salt loading on the development and progression of diabetic nephropathy in SDT fatty rats were evaluated. The effects of hyperglycemia and hypertension on the development and progression of diabetic nephropathy in SDT fatty rats using pharmacological approaches were also investigated.

UAE and serum BUN levels in unilateral nephrectomized SDT fatty rats increased from an early age compared with those in SDT fatty rats (Chapter 2: Figs. 1A and 1B). In histopathological examinations, a deterioration in glomerular and tubulointerstitial lesions was observed in unilateral nephrectomized SDT fatty rats (Chapter 2: Table 1). The rise in glomerular pressure due to the increase in renal blood

flow in remnant kidneys after unilaterally nephrectomy has been suggested to be a worsening factor. Unilateral nephrectomized SDT fatty rats showed elevations in blood lipids compared with SDT fatty rats. UAE is known to lead to elevations in blood lipids as a compensatory change (Peric & Peric 1983; Kasiske *et al*. 1988). Lipid-lowering agents have been shown to ameliorate injuries to reduced renal mass models of chronic renal failure (Kasiske *et al*. 1988). Abnormalities in the lipid metabolism of unilateral nephrectomized SDT fatty rats may also contribute to progressive renal injury.

There are several reports regarding the effects of unilateral nephrectomy on renal disease in STZ rats and Zucker fatty rats. However, a longer period of time is required for renal lesions to manifest in both models (Lopes *et al.* 2004; Kasiske *et al.* 1989). Difficulties in analyzing the pathology of diabetic nephropathy and developing novel drugs are considered to be mainly due to the complexity of the pathological mechanism, long-term pathological process and species differences between humans and animals, etc. The early onset of diabetic nephropathy in unilateral nephrectomized SDT fatty rats is an important component for mechanism analyses of diabetic nephropathy. Furthermore, the early onset is an advantage for the development of new therapies and new drugs for human diabetic nephropathy.

Diabetic patients reportedly have high sensitivity to salt and salt plays an important role in the control of blood pressure (Tuck *et al.* 1990). In Chapter 3, the effects of salt loading on blood pressure and renal function were investigated in SDT fatty rats. A significant increase in blood pressure was observed in salt-loaded SDT fatty rats at 14 weeks of age (Chapter 3: Fig. 7A). An increase in blood pressure was reportedly observed in other obese type 2 diabetic models, such as Zucker fatty rats and Wistar fatty rats, after salt loading (Suzuki *et al.* 1996; Reddy *et al.* 1992). The increase

in blood pressure was significant in SDT fatty rats compared with other obese type 2 diabetic animal models, suggesting that SDT fatty rats have a noticeably high salt sensitivity. A high incidence of salt-sensitive blood pressure responses were reportedly observed in patients with diabetic nephropathy (Krzysztof *et al*. 2005). These results suggest that SDT fatty rats may be used for the investigation of the relationship between diabetic nephropathy and high salt sensitivity.

Salt-loaded SDT fatty rats showed decreases in glomerular filtration function and a deterioration in pathological renal findings, including glomerulosclerosis and tubular and interstitial lesions (Chapter 3: Fig. 8D, Table 2). In addition to other renal findings, a marked thickening of arterioles was observed in histological examinations of kidneys (Chapter 3: Table 2). This change is suggested to be a hypertension-related finding. Salt-sensitive hypertension is closely related with the renal excretory function of sodium. There are several factors that modulate renal function for urinary sodium excretion (Hall *et al*. 1990; Coffman 2011). For example, the activation of the renin-angiotensin system increases tubular sodium reabsorption and leads to blood pressure elevation (Gonzalez-Villalobos *et al*. 2011, 2013; Kobori *et al*. 2003).

Another important factor influencing salt-sensitive hypertension is the renal sympathetic nervous system (Jacob *et al*. 2005; Foss *et al*. 2013). The antinatriuretic effect of increased renal sympathetic nervous system activity is mainly mediated by increased renin secretion, reduced renal blood flow, and increased renal tubular reabsorption (DiBona 2005).

The dysfunction in sodium excretion in the kidney leads to an increase in plasma volume. In general, BUN levels increase in renal disease; however, BUN levels in salt-loaded SDT fatty rats decrease (Chapter 3: Fig. 8A). The decrease in BUN levels in

salt-loaded SDT fatty rats may be caused by the dysfunction in sodium excretion in the kidneys of SDT fatty rats.

Innate and adaptive immune cells are also thought to be essential for the pathogenesis of salt-sensitive hypertension. The infiltration of T cells and macrophages in the perivascular space and kidney is observed in the salt-sensitive hypertensive model. Mice deficient in T cells and macrophages suppress the elevation of blood pressure in the salt-sensitive hypertensive model (Guzik *et al*. 2007; Ko *et al*. 2007). Reactive oxygen species and cytokines that are produced by T cells and macrophages cause sodium retention, resulting in salt-sensitive hypertension. Recently, sodium was reported as accelerating the differentiation of naïve CD4 positive T cells into T-helper 17 lymphocytes (Th17) (Kleinewietfeld *et al*. 2013; Wu *et al*. 2013). Th17 cells mainly produce the proinflammatory cytokine interleukin-17 (IL-17). IL-17 promotes sodium reabsorption via the upregulation of serum- and glucocorticoid-induced kinase-1 and sodium-hydrogen exchanger 3 at the proximal renal tubule in the kidney. Treatment of salt-sensitive hypertensive rats with an anti-IL-17 antibody significantly reduced the elevation in blood pressure (Amador *et al*. 2014). These findings indicate that IL-17 may participate in hypertension produced by a high-salt diet. Salt-loaded SDT fatty rats showed marked inflammatory cell infiltration in the kidney. SDT fatty rats also reportedly showed ocular inflammation in the uveal tract (Kemmochi *et al.* 2014). Further investigations will be required to determine the contribution of inflammation to the progression of diabetic nephropathy in SDT fatty rats after salt-loading.

In Chapter 3, SDT fatty rats were elucidated as having a noticeably high salt sensitivity and deterioration of renal disease and a variety of renal lesions that reflect the clinical picture of diabetic nephropathy were observed in salt-loaded SDT fatty rats. The

elevation in blood pressure and renal dysfunction due to the enhancement of salt sensitivity in SDT fatty rats are intriguing observations for the understanding of the pathogenesis and mechanism of diabetic nephropathy.

Current treatments for diabetic nephropathy are mainly focused on adequate control of blood glucose and hypertension. Thus, we investigated the effects of hyperglycemia and hypertension on the development and progression of diabetic nephropathy in SDT fatty rats. Blood glucose levels in SDT fatty rats were maintained at normal levels by administering a hypoglycemic drug. However, the effects on renal dysfunction were limited (Chapter 4: Fig. 14). Treatment with anti-hypertensive drugs also did not improve renal disease in unilateral nephrectomized SDT fatty rats (Chapter 5: Fig. 20). These data demonstrate that multiple factors affect the reduction in renal function in a complex manner and the control of only blood glucose or blood pressure will not lead to therapeutic effects that alleviate the decline in renal function in SDT fatty rats.

The control of glucose or blood pressure reportedly suppresses the development of diabetic nephropathy in other obese type 2 diabetic animal models, such as ZDF rats and OLETF rats (Gang *et al*. 2008; Giovanna *et al*. 2014; Mi *et al*. 2011). These findings indicate that worsening factors associated with diabetic nephropathy in SDT fatty rats are different from those in other obese type 2 diabetic models.

Epidemiological research revealed that dyslipidemia is one of the risk factors for the development and progression of diabetic nephropathy, in addition to hyperglycemia and hypertension (Perkins *et al*. 2003; Ravid *et al*. 1998; Appel *et al*. 2003). Hyperlipidemia is associated with glomerulosclerosis and a glomerular monocyte infiltrate. When cultured human mesangial cells were co-incubated with human LDL,

these cells demonstrated a greater level of tissue culture supernatant fibronectin and the increase in the expression of monocyte chemoattractant protein-1 mRNA, a monocyte specific chemotactic factor (Rovin & Tan 1993). Treatment of STZ diabetic mice with a high-fat diet synergistically aggravated real lesions, as indicated by the increase in UAE, macrophage infiltration, mesangial expansion and proinflammatory extracellular matrix-associated gene induction in glomeruli (Kuwabata *et al.* 2012). With regard to the treatment of dyslipidemia in patients with diabetes, there have been several clinical trials of anti hypercholesterolemic agents, including fibrates and statins, conducted. Fibrates and statins are representative drugs for dyslipidemia. In the Diabetes Atherosclerosis Intervention Study, fenofibrate reduced the worsening of UAE. (Ansquer *et al.* 2005). In the Collaborative Atorvastatin Diabetes Study, treatment with atorvastatin was associated with an improvement in eGFR (Colhoun *et al.* 2009). Results from clinical studies revealed the possibility that anti-hyperlipidemic agents have a beneficial effect on diabetic nephropathy through the improvement of albuminuria and loss of renal function.

With the early incidence of diabetes mellitus, dyslipidemia in SDT fatty rats was observed at younger ages. However, the control of blood glucose and blood pressure did not affect the dyslipidemia in SDT fatty rats (Chapter 4: Figs. 13A and 13B; Chapter 5: Figs. 18B and 18C). Continuous dyslipidemia may contribute to the development and progression of diabetic nephropathy in SDT fatty rats. In Chapter 4 and Chapter 5, SDT fatty rats were demonstrated as having different responses to hypoglycemic drugs and antihypertensive drugs compared with other diabetic animal models. The different characteristics of SDT fatty rats are considered to play a key role in elucidating the mechanism of diabetic nephropathy. There are numerous patients for whom the strict

control of blood glucose and blood pressure do not result in the suppression of progression of diabetic nephropathy. Therefore, unmet medical needs for these patients are extremely high. Further pathological analyses of SDT fatty rats have been suggested as possibly leading to the discovery of new therapeutic targets.

In conclusion, the present studies revealed that SDT fatty rats showed accelerated diabetic nephropathy due to unilateral nephrectomy and salt-loaded SDT fatty rats showed salt-sensitive hypertension, deterioration in renal function and characteristic pathological changes, such as a marked thickening of arterioles, in addition to other renal lesions that reflect the clinical picture of diabetic nephropathy. Furthermore, the worsening factors associated with diabetic nephropathy in SDT fatty rats were clarified as being different from those in other diabetic animal models. In the future, understanding the time-dependent pathological changes in diabetic nephropathy, identifying novel therapeutic targets, and exploring biomarkers will be important.

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