

**DIETARY SOY PROTEIN REDUCES EARLY RENAL DISEASE
PROGRESSION AND ALTERS PROSTANOID PRODUCTION IN
OBESE *fa/fa* ZUCKER RATS**

BY

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**A thesis submitted to the faculty of graduate studies in partial
fulfillment of the requirements for a degree of**

MASTER OF SCIENCE

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ABSTRACT

The growing epidemic of obesity in adults, as well as in children, is occurring throughout the world. It affects not only the individual's physical and mental health but carries substantial costs for the nation's economy. With the rising incidence of obesity and the metabolic syndrome, nephropathy associated with obesity also has increased. Soy protein can ameliorate disease in several models of chronic kidney disease. It also may alter the production of renal prostanoids, which may play a role in the glomerular hyperfiltration in obesity-associated nephropathy (OAN). Therefore, the effect of dietary soy protein on early disease progression and prostanoid production in OAN was examined in the obese *fa/fa* Zucker rat. Six-week-old male *fa/fa* and lean Zucker rats were offered diets *ad libitum* containing 17% protein from either soy protein or egg white protein for 8 weeks. Mean glomerular volume (MGV) and proteinuria were determined to assess early kidney changes and kidney function. Renal prostanoids [thromboxane B₂ (TXB₂), stable metabolite of TXA₂; 6-keto prostaglandin F_{1α} (6-keto PGF_{1α}), stable metabolite of PGI₂; and prostaglandin E₂ (PGE₂)] were determined by enzyme immunoassay. Renal protein and mRNA levels of cytosolic phospholipase A₂ (cPLA₂), cyclooxygenase-1 (COX-1), and COX-2 were determined by western immunoblotting and QRT-PCR. Feed consumption, body and kidney weights were significantly greater in *fa/fa* compared to lean rats. The *fa/fa* rats had 43% larger MGV and 169% higher proteinuria, reflecting early development of nephropathy. Soy protein feeding did not alter body weights or proteinuria but did result in 6% lower kidney weights

(g/100 g body weight) and 16% lower MGV. Prostanoid production in these kidneys was primarily due to COX-2 activity. Renal prostanoids were generally not altered by diet or genotype, however, renal 6-keto PGF_{1α} levels were lower in *fa/fa* rats fed soy protein. Renal TXB₂/PGE₂ ratios were not different in rats given soy protein compared to egg white protein while renal TXB₂/6-keto PGF_{1α} ratios were higher and 6-keto PGF_{1α}/PGE₂ ratios were lower, indicating that dietary soy protein reduces renal 6-keto PGF_{1α} levels. The attenuation of early nephropathy in *fa/fa* rats by dietary soy protein is associated with lower 6-keto PGF_{1α} levels. This may affect the glomerular hyperfiltration observed in OAN.

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TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	iv
LIST OF ABBREVIATIONS	vii
LIST OF TABLES	ix
LIST OF FIGURES	x
1. LITERATURE REVIEW	
1.1 Introduction	
1.1.1 A Worldwide Epidemic: Obesity	1
1.1.2 Childhood Obesity	3
1.1.3 Metabolic Syndrome	4
1.1.4 Obesity-Associated Nephropathy (OAN)	6
1.1.5 The Mechanism of OAN	11
1.2 Dietary Intervention in Renal Disease	
1.2.1 Fat and Renal Disease Progression	13
1.2.2 Protein and Renal Disease Progression	15
1.2.3 Dietary Soy Protein and Renal Disease	17
1.3 Prostanoids	
1.3.1 Production of Prostanoids from Cyclooxygenase Enzymes	20
1.3.2 Prostanoid Enzymes and Prostanoid Production in Renal Disease	23
1.3.3 Prostanoid Production and Dietary Interventions	25
1.4 The Obese fa/fa Zucker Rat	27

1.5 Hypotheses and Objectives	28
2. DIETARY SOY PROTEIN REDUCES EARLY RENAL DISEASE PROGRESSION AND ALTERS PROSTANOID PRODUCTION IN OBESE <i>fa/fa</i> ZUCKER RATS	
2.1 Abstract	30
2.2 Introduction	32
2.3 Materials and Methods	33
2.3.1 Animals and Diet	33
2.3.2 Glomerular Size	35
2.3.3 Immunoblotting	36
2.3.4 Prostanoid Production and COX Activity	37
2.3.5 Quantitative RT-PCR	38
2.3.6 Statistical Analysis	39
2.4 Results	39
2.5 Discussion	54
3. OVERALL DISCUSSION	57
4. STRENGTHS AND LIMITATIONS	63
5. FUTURE RESEARCH	64
6. REFERENCES	66
7. APPENDIX	
7.1 Mean Glomerular volume (MGV)	78
7.2 Measurement of Steady-State Protein Levels of Enzymes Involved in Prostanoid Production	

7.2.1 Lyophilization of Kidneys	79
7.2.2 Homogenization of Kidneys	80
7.2.3 Total Protein Determination	82
7.2.4 Western Immunoblotting	82
7.3 Measurement of Kidney Function	
7.3.1 Urine Creatinine	88
7.3.2 Serum Creatinine	89
7.3.3. Creatinine Clearance	91
7.4 Prostanoid Production and COX Activity	91
7.5 Effects of Dietary Soy Protein on Urine and Serum Parameters in Obese <i>fa/fa</i> Zucker rats	94

LIST OF ABBREVIATIONS

AA	arachidonic acid
APS	ammonium persulfate
BCA	bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumin
CLA	conjugated linoleic acid
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
cPLA ₂	cytosolic phospholipase A ₂
CTL	control diet
CVD	cardiovascular disease
Cytosolic cPLA ₂	cytosolic phospholipase A ₂ present in cytosolic fraction
DGLA	dihomo-gamma-linolenic acid
DM	diabetes mellitus
DM-2	type 2 diabetes mellitus
EPA	eicosapentaenoic acid
ERPF	effective renal plasma flow
ESRD	end-stage renal disease
<i>fa/fa</i>	obese <i>fa/fa</i> Zucker rat
FF	filtration fraction
GFR	glomerular filtration rate
IDV	integrated density value
IgG	immunoglobulin G
Lean	lean Zucker rat
mA	milliamps
MGA	mean glomerular area
MGV	mean glomerular volume
mRNA	messenger ribonucleic acid
NHANES	National Health and Nutrition Examination Survey
OAN	obesity-associated nephropathy
Particulate cPLA ₂	cytosolic phospholipase A ₂ present in particulate fraction
PBF	phosphate-buffered formalin
PBS	phosphate-buffered saline
PGE ₂	prostaglandin E ₂
6-keto PGF _{1α}	6-keto prostaglandin F _{1α}
PGI ₂	prostaglandin I ₂
PKD	polycystic kidney disease
PREVEND	Prevention of Renal and Vascular End Stage Disease study
QRT-PCR	quantitative reverse-transcriptase-polymerase chain reaction

rpm	revolutions per minute
RT-PCR	reverse-transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
TBS	tris base solution
TEMED	N,N,N',N',-tetramethylethylenediamine
tris-HCL	tris (hydroxymethyl) aminomethane-hydrochloric acid
TXA ₂	thromboxane A ₂
TXB ₂	thromboxane B ₂
WHO	World Health Organization
ZDF	Zucker Diabetic Fatty rats

LIST OF TABLES

Table 1	Body weight classifications in adults	2
Table 2	Risk factors for the metabolic syndrome	5
Table 3	The five stages of chronic kidney disease	9
Table 4	Diet formulation	34
Table 5	Effects of dietary soy protein on feed intake, body and kidney weights, proteinuria, and creatinine clearance in obese <i>fa/fa</i> Zucker rats	40
Table 6	Effects of dietary soy protein on endogenous prostanoid levels in kidneys of obese <i>fa/fa</i> Zucker rats	44
Table 7	Effects of dietary soy protein on in vitro steady-state prostanoid levels in kidneys of obese <i>fa/fa</i> Zucker rats	45
Table 8	Effects of dietary soy protein on total COX activity in kidneys of obese <i>fa/fa</i> Zucker rats	46
Table 9	Effects of dietary soy protein on COX-2 activity in kidneys of obese <i>fa/fa</i> Zucker rats	47

LIST OF FIGURES

Figure 1	Effects of dietary soy protein on mean glomerular volume (MGV) in obese <i>fa/fa</i> Zucker rats	42
Figure 2	Kidney cross sections stained with eosin and hematoxylin	43
Figure 3	Effects of dietary soy protein on TXB ₂ /6-keto PGF _{1α} ratio in kidneys of obese <i>fa/fa</i> Zucker rats	49
Figure 4	Effects of dietary soy protein on 6-keto PGF _{1α} /PGE ₂ ratio in kidneys of obese <i>fa/fa</i> Zucker rats	50
Figure 5	Effects of dietary soy protein on TXB ₂ /PGE ₂ ratio in kidneys of obese <i>fa/fa</i> Zucker rats	51
Figure 6	Effects of dietary soy protein on relative cytosolic and particulate cPLA ₂ , COX-1, and COX-2 protein expression	52
Figure 7	Effects of dietary soy protein on relative cPLA ₂ , COX-1, and COX-2 mRNA expression	53

1. Literature Review

1.1 Introduction

1.1.1 A Worldwide Epidemic: Obesity

The prevalence of obesity is increasing rapidly in many parts of the world. Over the last two to three decades, obesity has transformed from a relatively minor public health issue to a major threat to public health. This transformation is now increasingly being seen throughout the world. Hence, it seems reasonable to describe obesity as a public health crisis that severely impairs the health and quality of life of people.

In populations in the world, the prevalence of obesity is high in both men and women, as well as even in children. In Canada, 23% of adults are obese and the incidence of obesity is rising with 9% of children being considered obese (Statistic Canada, 2004). The growing obesity epidemic also affects direct and indirect costs for the nation's economy. Urgent action, therefore, is required to reverse current trends.

Obesity, defined as a body mass index (BMI) of 30 kg/m^2 and higher, is a complex condition that has its origin in the interaction between an individual's genetic factors, eating behavior, cellular metabolism, life style, and cultural background of the community as well as socio-economic status (**Table 1**). Obesity is associated with increased risk of a number of disorders and there is evidence to suggest a strong relationship between obesity and metabolic and cardiovascular risk factors such as high blood pressure, dyslipidemia, hyperinsulinemia and/or insulin resistance (Hall et al., 2002; Jong 2002). In

Table 1. Body weight classifications in adults¹

Body Mass Index (kg/m²)	Classification	Description
< 18.5	Underweight	Might be associated with health problems
18.5 – 24.9	Normal weight	Good weight for most people
25.0 – 29.9	Overweight	Increasing risk of developing health problems
≥ 30	Obese	High risk of developing health problems

¹ Adapted from Kim, 2004; World Health Organization, 2000

addition, obesity also contributes to psychological and social burdens such as social stigma, low self-esteem, reduced mobility, and a poorer quality of life.

1.1.2 Childhood Obesity

A rapid increase in the prevalence of obesity among children has been documented in many countries. It is recently estimated that 7% of children in the world are obese, with the U.S. having the highest prevalence (Speiser et al., 2005).

Since children's development varies with age, and boys and girls develop at different rates, the use of BMI to assess body weight in children requires growth and gender considerations. Therefore, BMI values for children and youth are specific to both age and gender. The term obesity in children and youth as defined by Centers for Disease Control and Prevention (CDC) refers to children between the ages of 2 and 18 years with BMIs equal to or greater than the 95th percentile (Kuczmarski et al., 2000).

The national surveys from the U.S. done between 1963 and 1991 found that since 1980 the number of overweight children has doubled and the number of overweight adolescents has tripled (Troiano et al., 1995). Moreover, the Bogalusa Heart Study reported that the state of being overweight or obese continues to persist into young adulthood, where 58% of overweight adolescent children remained overweight as young adults (Jiang et al., 1995).

It has been reported that there was a significant increase in the prevalence of hypertension and dyslipidemia in overweight adolescents when followed up in

later years (Srinivasan et al., 1996). A significant increase in mortality, especially that due to cardiovascular disease, has also been reported in long-term follow-up studies of overweight children (Hoffmans et al., 1988; Mossberg, 1989; Must et al., 1992). The impact of childhood obesity on psychological morbidity, such as low self-esteem, behavioral problems, and obstructive sleep apnea, which were once considered adult diseases, are now being observed in children with obesity (Reilly et al., 2003). Therefore, the growing obesity crisis will need to be addressed at individual, family, community, national and global levels to ameliorate the health consequences of obesity in children.

1.1.3 Metabolic Syndrome

The term metabolic syndrome, also called “syndrome X”, is used to describe the co-existence of several metabolic characteristics. The metabolic syndrome, as defined in the Third Report of the National Cholesterol Education Program (NCEP, 2002), is defined as the presence of three or more of the following risk determinants (**Table 2**): (1) abdominal obesity (increased waist circumference); (2) elevated triglycerides; (3) low HDL cholesterol; (4) hypertension; and (5) elevated fasting glucose.

The metabolic syndrome is recognized as an independent risk factor for the development of type 2 diabetes mellitus (DM) (Lorenzo et al., 2003). Since type 2 DM takes years to develop after signs of the metabolic syndrome are present, the pre-diabetic state of the metabolic syndrome can be considered as a warning sign (Goldstein, 2003). Therefore, it is important to initiate interventions at this

Table 2. Risk factors for the metabolic syndrome¹

Metabolic Syndrome Risk Factor	Defining Level for Men	Defining Level for Women
Abdominal Obesity (or waist circumference)	> 102 cm	> 88 cm
Triglycerides	≥ 150 mg/dl	≥ 150 mg/dl
HDL Cholesterol ²	< 40 mg/dl	< 50 mg/dl
Blood Pressure	≥ 130/85 mmHg	≥ 130/85 mmHg
Fasting Glucose	≥ 110 mg/dl	≥ 110 mg/dl

¹ Adapted from Chen et al., 2004 and National Cholesterol Education Program, 2002

² HDL = high-density lipoprotein

early stage of disease. Moreover, the metabolic syndrome is not only a health concern for the adult, but is now becoming a problem for young people as well.

In 2004, Lambert et al. reported based on a study conducted in Quebec, the overall prevalence of the metabolic syndrome in youth in Canada was 11.5%. Considering the average age of the participants in that study, 9, 13, or 16 years of age, this prevalence is of great concern. The metabolic syndrome is associated with an increased risk of chronic kidney disease and its association with kidney disease has been reported in childhood as well (Abrass, 2004). Obese children, with an average age of 12.9 years, had a significantly higher urinary albumin to creatinine ratio compared to children of normal weight. Among the obese children, the presence of several cardiovascular risk factors, such as hyperinsulinemia, impaired glucose tolerance, and hypercholesterolemia, were all associated with a significantly higher urinary albumin to creatinine ratio compared to those that did not have these risk factors (Csernus et al., 2005). It is evident from these studies that strategies to prevent and/or delay chronic kidney disease must be started before a clinical disease such as DM is diagnosed. Specifically, these strategies need to be initiated in the early stage of obesity.

1.1.4 Obesity-Associated Nephropathy (OAN)

The role of obesity in DM and diabetic nephropathy is well-recognized. However, the impact of obesity on the kidney has not received much attention so far. Therefore, this thesis is focused on bringing attention to the impact of obesity on the progression of kidney disease.

As obesity is associated with the two most common causes of end stage renal disease (ESRD), which are DM and hypertension, it seems likely that obesity may greatly increase the risk of ESRD. The Okinawa Dialysis Study registry found the cumulative incidence of ESRD to increase from the lowest BMI quartile (2.5 case/1,000) to the highest BMI quartile (5.8 case/1,000) in a screening program (Iseki et al., 2004).

In 2000, Praga et al. demonstrated the long-term effects of obesity on the kidney in a follow-up study of 73 patients who had undergone unilateral nephrectomy. Fourteen of the 73 patients were obese at the time of nephrectomy in that study. At the 20 years follow-up, it was found that most of the non-obese subjects but only 30-40% of the obese subjects still had normal renal function. In 2001, Bonnet et al. reported that the presence of an elevated BMI (≥ 25 kg/m²) at the time of renal biopsy in 162 patients with IgA nephropathy was correlated with the severity of pathological abnormalities and with clinical progression to ESRD. In 1986, Kasiske et al. investigated the type of renal abnormalities seen in obese patients. This study compared the clinical and histological patterns in 17 patients with massive obesity (mean weight 126 kg) and marked proteinuria with control group of 34 patients with similar clinical presentation but normal body weight (mean weight 68 kg). Although urinary protein excretion was similar in the two groups, serum albumin was higher in the obese subjects. With respect to the histological data, most of the obese patients had a focal glomerulosclerosis while most of the control subjects had a minimal change nephritic syndrome.

In the initial stages of renal disease, glomerular filtration rate (GFR) tends to increase due to increased glomerular capillary pressure, called glomerular hyperfiltration (Mogensen et al., 1990) and kidney damage is evidenced by structural and/or functional abnormalities (National Kidney Foundation, 2002). Five stages of chronic kidney disease have been identified (**Table 3**). Stage 5, also known as ESRD, represents complete kidney failure. At this point, dialysis or transplantation is required for patient survival (National Kidney Foundation, 2002). In 2002, the prevalence of ESRD in the U.S. was 1,435 per million population (USRDS, 2004).

It has previously been shown that an increased BMI is associated with microalbuminuria (Ribstein et al., 1995). A study to determine the impact of obesity on renal function in the general population was completed in a sub-analysis of the Prevention of Renal and Vascular End Stage Disease study (PREVEND), which was initiated to study the impact of microalbuminuria on renal and cardiovascular risk in the general population (Hillege et al., 2001; Pinto-Sietsma et al., 2000). In the general population aged 28-75 years old, microalbuminuria was found to be present in 16.4% of subjects with DM, 11.5% of those with hypertension and 6.6% of healthy subjects who were not known to have either DM or hypertension (Hillege et al., 2001).

So what causes microalbuminuria in these non-diabetic and non-hypertensive subjects? Data including two 24-hour urine collections to measure 24 hour urinary albumin excretion of 8592 subjects were examined to address this question (Pinto-Sietsma et al., 2000). Microalbuminuria was defined

Table 3. The five stages of chronic kidney disease¹

Stage	Characteristics	GFR² (mL/min/1.73 m²)	Albumin Excretion
Stage 1	Glomerular hyperfiltration & hypertrophy with normal or increased GFR	≥ 90	May be increased
Stage 2	Expanding mesangium with mild decreased GFR	60-89	< 30-300 mg/24hr
Stage 3	Moderate decreased GFR	30-59	30-300 mg/24hr (microalbuminuria)
Stage 4	Severe decreased GFR	15-29	> 300 mg/24hr (macroalbuminuria)
Stage 5	ESRD ³	< 15	Decreasing

¹ Aadapted from Gillis, 1998; Mogensen et al., 1987; National Kidney Foundation, 2002

² GFR = glomerular filtration rate

³ ESRD = end-stage renal disease

according to the classical criterion of 30-300 mg albumin excreted per 24 hour (**Table 3**). Since data from 542 subjects were excluded from this analysis because urinary albumin excretion was not reliable or data was otherwise missing, the data of 8050 subjects were used. BMI was classified as normal body mass ($<25 \text{ kg/m}^2$), overweight ($25\text{-}29.9 \text{ kg/m}^2$), or obese ($>30 \text{ kg/m}^2$). In men, about 47% were overweight and 14% were obese, while among women, 34% were overweight and 16% were obese. In both genders, a higher BMI was associated with greater 24-hour urinary albumin excretion. In men, the prevalence of microalbuminuria increased from 9.5% in those with a normal body weight to 18.3% in those who were overweight and to 29.3% in those with obvious obesity. In women, these percentages were 6.6, 9.2, and 16.0%, respectively. Multivariate analysis also showed that BMI was independently associated with urinary albumin excretion at increasing BMI in women (Verhave et al., 2002).

As GFR starts to decrease, urinary albumin excretion rises further to macroproteinuric ranges. In non-diabetic subjects, creatinine clearance was higher in subjects with a high normal albumin excretion (15-30 mg/day) than in controls (albumin excretion 0-15 mg/day) and was still elevated in microalbuminuric (albumin excretion 30-300 mg/day) persons while it was lower in the macroproteinuric (albumin loss $> 300 \text{ mg/day}$) subjects (Pinto-Sietsma et al., 2000). This pattern is similar to that described in DM, with initial glomerular hyperfiltration, followed by gradual loss of renal function. Therefore, this suggests that the higher risk for microalbuminuria is associated with a higher risk for

glomerular hyperfiltration and ultimately impaired glomerular filtration in non-diabetic subjects. These data are compatible with renal function studies, which have shown that obese subjects have an elevated renal blood flow and GFR (Jong et al., 2002).

The other major outcome of chronic kidney disease, aside from ESRD, is cardiovascular disease (CVD) (National Kidney Foundation, 2002). CVD is also the leading cause of death in patients with kidney failure. Dialysis patients from 25 to 35 years of age have a mortality risk from CVD that is 500 fold higher compared to the general population (National Kidney Foundation, 2002).

Even though the risk of developing kidney failure depends on the level of kidney function and the rate of decline of kidney function (National Kidney Foundation, 2002), earlier detection and appropriate interventions may delay kidney disease and the rate of decline of kidney function. This could potentially delay the diagnosis of ESRD and subsequent risk of CVD.

1.1.5 The Mechanism of OAN

What then is the mechanism behind the OAN? Hyperfiltration in non-diabetic obese individuals occurs because of increased transcapillary hydraulic pressure due to dilatation of the afferent arteriole without a change in diameter of the efferent arteriole. In 1995, Ribstein et al. studied non-diabetic subjects with BMIs between 27 and 40 (kg/m^2) and reported that GFR and effective renal plasma flow (ERPF) were increased in overweight compared with lean subjects. Chagnac and colleagues did a similar study demonstrating an increased GFR,

ERPF, filtration fraction (FF), and urinary albumin excretion rate in obese subjects. The data show renal hyperfiltration in non-diabetic obese individuals. Moreover, the impact of elevated BMI on renal hemodynamics is not limited to overt obesity, as even subjects with BMI <30 kg/m² have elevated FF from a higher GFR relative to ERPF suggesting higher glomerular pressure (Bosma et al., 2004).

Hormonal factors also may be involved in these mechanisms. Insulin resistance, which is mainly present in obesity, could be one of the mechanisms as insulin resistance induces systemic and intraglomerular hypertension (Tucker et al., 1992) as well as mesangial hypertrophy and increased mesangial matrix production (Abrass et al., 1999).

Leptin is a small peptide hormone secreted by adipocytes that regulates food intake. In obese individuals and in patients with impaired renal function, serum leptin levels were increased (Considine et al., 1996). In glomerular endothelial cells, leptin stimulates cellular proliferation and transforming growth factor- β 1 synthesis (Wolf et al., 1999). Wolf et al. (1999) found out that infusion of leptin in normal rats for 3 weeks resulted in glomerulosclerosis and proteinuria.

The role of inflammation in obesity may be another factor in the development of OAN. It has been reported that C-reactive protein (CRP) levels are elevated with BMI suggesting a state of low-grade systemic inflammation (Visser et al., 1999; Yudkin et al., 1999). Similarly, in 2002, Jong et al. also found that CRP is higher in men and women with elevated BMI compared to individuals with BMI <25 kg/m².

Obesity leads to glomerular hyperfiltration, increased urinary albumin loss and a progressive loss of renal function, associated with a focal segmental glomerulosclerosis. This is present not only in subjects with renal disease, but also in obese subjects without chronic disease.

1.2 Dietary Intervention in Renal Disease

1.2.1 Fat and Renal Disease Progression

The effect of different levels and sources of dietary fat have been studied in several types of renal diseases. Reducing the level of dietary fat retards disease progression in animal models of both the CD1-*pcy/pcy* mouse and the Han:SPRD-*cy* rats (Lu et al., 1999; Jayapalan et al., 2000). Lu et al. (1999) showed that male CD1-*pcy/pcy* mice fed a 20% soybean oil diet for 130 days compared to a 4% soybean oil diet had higher kidney weight relative to body weight suggesting the potential detrimental effects of high dietary fat. Similarly, in male Han:SPRD-*cy* rats, consuming a 20% soybean oil for 6 weeks compared to a 5% soybean oil resulted in increased kidney weights, kidney water content, cyst scores and serum creatinine, which indicate kidney disease progression and worsened renal function (Jayapalan et al., 2000). These studies show that a low fat diet compared to a high fat diet slows disease progression in animal models of renal cyst disease.

Different fat sources, such as flaxseed, fish oil, and conjugated linoleic acid (CLA), have been studied in several rodent models of renal disease. Flaxseed, a rich source of n-3 fatty acids, has been demonstrated to ameliorate the chronic

interstitial nephritis associated with renal cystic disease in Han:SPRD-*cy* rats (Ogborn et al., 1999). In this study, flaxseed-fed rats had lower serum creatinine, less cystic change, and less renal fibrosis than controls. In 1990, Yamaguchi et al. reported that fish oil, also containing higher amounts of n-3 fatty acids, also slowed early cyst formation in cystic renal disease (Yamaguchi et al., 1990). One previous study from our laboratory showed that long-term feeding of dietary fish oil did not improve survival in *pcy* mice (Aukema et al., 1992). However, in the Han:SPRD-*cy* rat, the detrimental effects of a high fat diet in early renal injury were ameliorated by fish oil (Lu et al., 2003). Moreover, 8 weeks of CLA feeding to the same rat model, the Han:SPRD-*cy* rat, significantly reduced renal inflammation and renal fibrosis (Nitschmann et al., 2001; Ogborn et al., 2003).

In 1991, Kasiske et al. reported that the source of dietary fatty acid consumed altered the fatty acid profile in the kidney of obese *fa/fa* Zucker rats. After 24 weeks of dietary intervention, 34 week old obese *fa/fa* Zucker rats fed a 25% fish oil diet had higher levels of renal n-3 fatty acids as reflected by increased eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels while arachidonic acid (AA) levels were decreased. Obese *fa/fa* Zucker rats fed 25% sunflower oil had higher levels of renal n-6 fatty acids as reflected by increased linoleic acid while EPA levels were decreased; both the fish oil and sunflower oil groups had reductions in glomerulosclerosis. In 1999, Wheeler et al. reported a similar study using 40 obese *fa/fa* Zucker rats that had undergone unilateral nephrectomy in order to exacerbate lipid abnormalities and accelerate proteinuria at 10 weeks of age. Rats were divided into three groups. One group

was given the control diet while the other two groups received a diet supplemented with either 14% fish oil or 14% melted beef tallow for 32 weeks. Rats fed fish oil had significantly lower mean plasma cholesterol, triglyceride levels, and less proteinuria than control or beef tallow fed rats. Thus, dietary fat can have a significant effect on progression of kidney disease.

1.2.2 Protein and Renal Disease Progression

The role of dietary protein intervention also has been the issue of current research on renal disease progression. It has been demonstrated that both the levels and sources of protein are associated with renal disease progression. Research has shown that high protein diets have a detrimental effect on renal structure and accelerate renal disease progression (Brenner et al., 1982; Ogborn et al., 1995; Tomobe et al., 1994; Williams et al., 1987). In 1982, Brenner et al. discovered that excessive protein intake causes glomerular hyperfiltration, glomerular hypertension, and deterioration of kidney function in the renal ablation model of chronic kidney disease. It has been suggested that individuals who suffer from renal insufficiency should restrict protein intake to lower the kidney work load.

In *pcy* mice, a diet with 6% casein compared to 25% casein resulted in less renal enlargement and lower total cysts (Aukema et al., 1992). In the Han:SPRD-cy rat model, a diet with 8% casein compared to 20% casein resulted in increased survival time and a reduction in total cyst volume when compared to non-affected animals. Mean serum creatinine and urea levels were significantly

lower in the low protein fed rats when compared to the 20% protein fed rats (Ogborn et al., 1995).

Several human studies also have shown similar results. In 1987, Viberti et al. established that low protein diets in healthy humans were associated with lower renal plasma flow, lower GFR, and less albumin excretion. In 1983, Maschio et al. examined the effect of low protein on renal patients ranging from 15 to 68 years of age. This study demonstrated that dietary protein restriction was effective for slowing progression of renal failure in most patients. Similarly, in 1983, Alverstand et al. reported that protein restriction of 15 to 20 g/day in middle aged patients with renal insufficiency lowered serum creatinine levels. A clinical trial was conducted by the Modification of Diet in Renal Disease Study Group to determine if protein restriction influences the progression of renal disease. This study demonstrated a marginal benefit of protein restriction (0.58 g/kg/day) compared to the typical protein intake (1.3 g/kg/day) in patients suffering from renal insufficiency. In contrast, Klahr et al. (1994) showed no benefit of a very low protein diet (0.28 g/kg/day) in those patients with severe renal disease.

Several meta-analysis have examined the effect of dietary protein restriction on renal function and disease progression have been reported (Kasiske et al., 1998; Pedrini et al., 1996). The results of these studies showed that dietary protein restriction slows the progression of disease in both diabetic and non-diabetic renal diseases.

Studies also have suggested that not only does a low protein diet slow the progression of renal disease but that the type of protein also may play an

important role. In 1990, Kontessis et al. found that vegetable-based protein diets for 3 weeks had no significant effects on GFR, renal vascular resistance or renal plasma flow in healthy individuals. However, when given animal protein, GFR and renal plasma flow rose and renal vascular resistance fell. One such plant-based protein that has been examined in different models of renal disease is soy protein.

1.2.3. Dietary Soy Protein and Renal Disease

Recently, research investigating the role of soy in renal disease and in other chronic diseases has increased dramatically. Especially in the area of chronic kidney disease, a soy based diet appears to attenuate the progression of disease. Soy protein has been recognized as having potential roles in the prevention and treatment of chronic diseases such as cardiovascular disease, cancer, osteoporosis, as well as kidney disease (Messina, 1995).

In 1998, Anderson et al. studied the effects of soy protein on renal function and proteinuria in patients with type 2 DM. It was reported that eating 50% of dietary protein as soy protein for 8 weeks had no distinct effect on renal function or proteinuria in these patients, but it was associated with a significant reduction in serum cholesterol and triglycerides.

In 1987, Williams et al. studied the effects of protein source as well as protein amount using the renal ablation model, an accepted and widely used model of chronic renal disease. Rats were given either a 12% or 24% soy protein diet or a 12% or 24% casein diet for 12 weeks. Soy protein demonstrated

beneficial effects on the renal ablated kidney, irrespective of the amount of protein, 12% or 24% protein intake. Soy fed rats demonstrated less proteinuria, less hypertrophy, less glomerular sclerosis and a lower mortality rate than casein fed rats. This study indicates that vegetable protein has a less detrimental effect on renal tissue than animal protein.

Subtotally nephrectomized rats given 24% soy protein-based diets for 10-13 weeks were shown to have improved survival rate, reduced proteinuria, renal hypertrophy, and renal histological damage compared with rats given 24% casein diet (Williams et al., 1987). Soy protein feeding in Han:SPRD-cy rats for 6 weeks reduced the number of renal cysts, fibrosis, macrophage infiltration, renal tubular cell proliferation, and apoptosis when compared to a casein based diet (Ogborn et al., 1998). Similar results were also found in the *pcy* mouse. In this model of renal cyst disease, after 13 weeks of feeding soy protein, animals had lower kidney weights, reduced cyst scores, and less kidney water, indicating a slower progression of disease (Aukema et al., 1999). Long-term feeding of dietary soy protein compared with casein increased life span and decreased renal pathology in Fischer 344 rats (Iwasaki et al., 1988; Shimokawa et al., 1993). In db/db mice, commonly used as a model of type 2 DM, improved glomerular macromolecular permeability and slower development of renal disease were detected after consuming a either 12% or 20% soy protein diet for 21-26 weeks (Teixeira et al., 2003). Moreover, in rats with chronic nephritic syndrome, soy protein feeding improved renal function and reduced renal damage and inflammatory cytokines (Tovar et al., 2002; Pedraza-Chaverri et al., 2004). In weanling Han:SPRD-cy

rats given either 20% soy protein or 20% casein based diets for 8 weeks, soy fed rats had lower serum creatinine, lower urinary ammonium excretion, reduced renal cysts, renal fibrosis, macrophage infiltration, and renal tubular cell proliferation (Ogborn et al., 1998).

In 1999, Aukema et al. examined the effects of different dietary protein sources and levels on early disease progression in both male and female CD1-*pcy/pcy* mice, a model of renal cyst disease. Ten-week-old CD1-*pcy/pcy* mice were fed either a soy protein diet or casein diet at a level of 6% or 17.4% for 13 weeks. The results with respect to protein sources showed that mice fed the soy protein diet had lower relative kidney weights, lower cyst scores, less kidney water content, and reduced serum urea compared to animals fed casein. With respect to protein levels, dietary protein reduction resulted in lower relative kidney weights, lower cyst scores, less kidney water content, and less serum urea in this rodent model. Therefore, it was concluded both dietary protein source and level significantly affect renal disease progression in CD1-*pcy/pcy* mice with the effects of dietary soy protein being most pronounced in female animals fed the low protein diet and the effects of protein reduction being most pronounced in animals fed soy protein diet.

Studies from our lab have documented that dietary soy protein compared with casein delays disease progression at the very early stage of renal disease. Soy protein feeding resulted in reduced cyst growth and renal fibrosis as early as 1 to 3 weeks after it was fed to weanling Han:SPRD-*cy* rats (Fair et al., 2004). Dietary soy protein both in the maternal and post-weaning diet reduced cyst

growth, cell proliferation, inflammation, and proteinuria (Peng, 2005). These studies demonstrate that early dietary soy protein intervention retards renal disease progression in several models of renal disease.

1.3 Prostanoids

1.3.1 Production of Prostanoids from Cyclooxygenase Enzymes

The kidney is a rich source of prostanoids which regulate renal hemodynamics, water and solute transport, and renin secretion (Imig, 2000). Altered prostanoid production has been shown in several diseased animal models (Perazella, 2002; Castellani et al., 2003). However, the effects of dietary soy protein on prostanoid production in OAN are not known.

The term, 'eicosanoids', refers to all the oxygenated products synthesized from 20-carbon polyunsaturated fatty acids (PUFAs) (Rahman et al., 1987; Smith and Murphy, 2002). These 20-carbon PUFAs include AA, dihomo- γ -linolenic acid (DGLA), and EPA. There are three major pathways leading to eicosanoid production including the cyclooxygenase (COX), lipoxygenase, and epoxygenase pathways (Vane et al., 1998; Williams et al., 1987).

Products of the COX pathway give rise to prostaglandins (PGs) and thromboxanes (TXs) which are collectively termed prostanoids. The lipoxygenase pathway leads to the formation of hydroperoxy eicosatetraenoic acids, hydroxy eicosatetraenoic acids, and leukotrienes. The epoxygenase pathway leads to the formation of epoxyeicosatrienoic acids. While all three pathways are present in

the kidney, the COX pathway is the major pathway for AA metabolism (Vane et al., 1998) and is the focus of the present study.

Of the 20 carbon fatty acids, there is usually a higher amount of AA in membrane phospholipids compared to either DGLA or EPA. The COX pathway has a higher specificity for AA than for the latter two fatty acids. Under normal circumstances, prostanoids derived from AA seem to be the most important. Series 1, series 2, and series 3 prostaglandins are derived from DGLA, AA, and EPA, respectively. Prostanoids derived from EPA tend to oppose or have weaker effects than those derived from AA (Dupont, 1990). The prostanoids coming from DGLA tend to have anti-inflammatory as well as anti-proliferative properties and, therefore, milder biological effects compared to AA derived prostanoids (Fan & Chapkin, 1998).

There are two isoforms identified, COX-1 and COX-2, both of which are inducible; COX-1 is present in nearly all cell types, while COX-2 is highly expressed in many tissues in response to inflammation (Aukema et al., 2002; Harris, 2000). COX-2 protein expression has been found in the macula densa/cortical thick ascending loop of Henle and medullary interstitial cells in the kidney of mice, rats, rabbits, and dogs (Harris, 2000). Previous studies on COX-2 localization in the human kidney reported expression in podocytes and arteriolar smooth muscle cells and it has also been shown to be expressed in the macula densa and medullary interstitial cells (Komhoff et al., 1997; Komhoff et al., 1999; Nantel et al., 1999). COX-1 gene expression in rat kidney is found in glomerular mesangial cells, distal convoluted tubule, connecting tubule, cortical, and

medullary collecting ducts, while COX-2 expression is found in glomeruli, cortical thick ascending limb, macula densa, and medullary interstitial cells (Harris, 2000).

Cytosolic phospholipase₂ (cPLA₂) releases AA from membrane phospholipids. AA is converted to prostaglandin H₂ by COXs, with subsequent conversion to the prostanoids such as prostaglandin I₂ (PGI₂), prostaglandin E₂ (PGE₂), and thromboxane A₂ (TXA₂).

COX is the enzyme that catalyzes the first steps in the production of PGs and TXs from AA. Products of this pathway are denoted by a numerical subscript '2' that represents the number of double bonds. PGs and TXs of this 2 series are the most common prostanoids formed (Smith and Murphy, 2002). Prostanoids containing one double bond are derived from DGLA, therefore, form the '1' series of eicosanoids. Prostanoids containing three double bonds derived from EPA form '3' series of eicosanoids.

Prostanoids are not stored by cells, but they are synthesized and released rapidly in response to extracellular hormonal stimuli where they act as local hormones since they are rapidly inactivated in the circulation (Smith and Murphy, 2002). With respect to the kidney, PGI₂ and PGE₂ are potent vasodilators that increase GFR, while TXA₂ is a potent vasoconstrictor that decreases GFR (Fitzgerald et al., 1987; Harris, 2002; Needleman et al., 1986).

1.3.2 Prostanoid Enzymes and Prostanoid Production in Renal Disease

In 2004, Dey et al. found that the protein expression of COX-1 did not differ in renal microvessels of male lean and obese Zucker diabetic fatty rats (ZDF) at the age of 20-21 weeks, while COX-2 expression was increased in renal microvessels of obese ZDF rats compared to lean rats. This study also reported that urinary excretion of TXB₂ and 6-keto PGF_{1α} were significantly higher in obese rats, but PGE₂ was lower in obese rats compared to lean rats. Rofecoxib, a COX-2 inhibitor, was given to the obese rats in their drinking water (10 mg/kg/day) for 3 weeks. However, this treatment did not alter urinary excretion of TXB₂ or PGE₂, but it reduced urinary excretion of 6-keto PGF_{1α} and ameliorated the glomerulosclerosis observed in ZDF rats (Dey et al., 2004). This study, therefore, indicates that COX-2 is involved in renal disease progression in ZDF rats as inhibiting this enzyme ameliorates glomerular injury in this obese rat model.

Similarly, in 2005, Komers et al. also found that COX-2 protein expression was higher in renal cortical tissue of male ZDF rats compared to lean Zucker rats at 4 weeks of age. Moreover, this higher expression of COX-2 became more prominent at 12 weeks of age. However, COX-1 protein expression did not differ between ZDF rats and lean rats at 4 weeks of age, but it was lower in ZDF rats at 12 weeks of age compared to lean rats. In ZDF rats, urinary excretion of PGE₂ and TXB₂ was significantly increased compared to lean rats at 12 weeks. ZDF rats had higher kidney weights at 4 and 12 weeks compared to lean rats. Even though creatinine clearance was not different between ZDF and lean rats, urinary

weeks. When wortmannin, an insulin-signaling intermediate, was given by intraperitoneal injection (100 µg/kg body weight) to additional groups of 12 week old ZDF and lean Zucker rats to examine the role of hyperinsulinemia on renal COX-2, ZDF rats treated with wortmannin had a lower TXB₂ excretion compared to vehicle-treated ZDF rats.

In 2005, Xu et al. reported that COX-2 messenger ribonucleic acid (mRNA) and protein expression were higher in the kidney cortex of 23 week old male *fa/fa* Zucker rats compared to lean control Zucker rats. Treatment with losartan (100 mg/L in the drinking water), an angiotensin II type 1 receptor blocker for 4 months attenuated the rise in COX-2 observed in the obese Zucker rats. However, COX-1 mRNA expression was not altered in any group of that study.

Studies from our laboratory have demonstrated that enzymes involved in prostanoid metabolism are altered in kidney disease and they seem to be altered in a different manner depending on the types of animal model, the age of the animal, and the specific area of the kidney that is being studied (Aukema et al., 2002; Herchak, 2005; Peng, 2005; Warford, 2003).

In the obese *fa/fa* Zucker rat, higher protein levels of cPLA₂ in cytosolic and particulate fractions and COX-2 in particulate fractions were observed at 14 weeks of age compared to lean Zucker rats (Herchak, 2005; Warford, 2003). Other studies from our lab using a mouse model of polycystic kidney disease indicate that protein expression of cPLA₂ in both the cytosolic and particulate fractions is higher in diseased male and female mouse kidneys compared to controls at 180 days of age (Aukema et al., 2002). Alterations in the COX

enzymes in these rodent models of polycystic kidney disease also were detected. COX-1 protein expression is higher in 180 day old male and female diseased mice kidneys and in 70 day old male diseased rat kidneys compared to controls. However, COX-2 protein levels were lower in diseased male rat kidneys compared to controls at 70 days of age (Aukema et al., 2002). These studies suggest that alterations in prostanoid production are important in the pathogenesis of renal disease.

1.3.3 Prostanoid Production and Dietary Interventions

Several studies have been carried out in order to determine whether dietary interventions modify renal hemodynamics through effects on renal prostanoids. The purpose of one such study was to determine whether dietary protein intake could exert effects on glomerular prostanoid production in rats with remnant kidneys (Stahl et al., 1987). Rats were placed on either a high protein (50%) or a low protein (8.7%) diet for 2 weeks. Higher proteinuria, GFR, and glomerular PGE₂, PGI₂, and TXA₂ production were found in rats on the high protein diet compared to low protein diet.

Another study demonstrated that dietary protein intake modulated glomerular prostanoid production in both normal and diseased rats (Don et al., 1989). Normal and diseased rats were offered either a high protein diet (40%) or a low protein diet (8.5%) for 10-14 days. Glomeruli were isolated and incubated in the absence and presence of AA, then prostanoid production was measured. In the absence of AA, glomerular production of PGE₂ and TXA₂ was significantly

greater in rats on the high protein diet. In the presence of AA, glomerular production of PGE₂ and TXA₂ also was greater in animals fed the high-protein diet suggesting that glomerular COX activity was augmented. Moreover, enalapril, an angiotensin-converting enzyme inhibitor, was used to investigate the potential role of the renin-angiotensin system in the dietary protein-induced modulation of glomerular prostanoid production in normal rats. Results demonstrated that enalapril attenuated the dietary protein-induced augmentation in glomerular production of PGE₂ and TXA₂ and it was concluded that dietary protein modulates glomerular PGE₂ and TXA₂ synthesis in the rats.

Altering the protein source is also related to alteration in renal hemodynamics, possibly via altering prostanoid production. In one study, healthy individuals were given either an animal protein or a soy protein diet for 3 weeks, and these two diets contained the same amount of total protein (Kontessis et al., 1990). Results showed that protein intake did not differ between groups, but GFR and urinary PGI₂ were significantly higher in individuals on the animal protein diet than the soy protein diet. Another study also showed that 1-3 weeks of soy protein feeding compared with casein ameliorated the suppression of PGE₂ production in the early stage of renal disease in the Han:SPRD-cy rat model of PKD, (Fair et al., 2004). In the same rat model, but at a later stage of the disease, dietary soy protein lowered the production of TXA₂ and PGI₂ (Peng, 2005).

The effects of plant protein compared to animal protein based diets on renal prostanoid production may be analogous to the effects of low compared to high

protein diets, i.e. ameliorating the changes in renal prostanoid production, hyperfiltration and renal disease progression.

1.4 The Obese *fa/fa* Zucker Rat

The obese *fa/fa* Zucker rat is a model of obesity and the metabolic syndrome. This rat model has a mutation in the leptin receptor resulting in hyperphagia and increased body size. Therefore, this model also develops symptoms of the metabolic syndrome including hypercholesterolemia, hyperinsulinemia, and hypertriglyceridemia observed in human obesity and is sometimes used as a model of pre-diabetes (Bray, 1977; Kasiske et al., 1985; Phillips et al., 1999).

The *fa/fa* Zucker rat also develops renal disease and ultimately dies of kidney failure. In 1985, Kasiske et al. demonstrated the progression of renal damage and albuminuria, as well as renal functional changes in obese male *fa/fa* Zucker rats at various ages. By 14 weeks of age, glomerular mesangial matrix expansion was significantly greater in *fa/fa* rats compared to lean rats followed by a rapid increase in albuminuria. By 28 weeks of age, focal glomerulosclerosis was evident and it was extensive at 68 weeks of age in *fa/fa* rats.

In 2000, Coimbra et al. studied age-related renal changes in male obese *fa/fa* Zucker rats at 6, 10, 14, 18, 40, and 60 weeks of age as well as lean Zucker rats as the controls at 6, 14, and 40 weeks of age. Glomerular hypertrophy was evident in *fa/fa* rats at 14 weeks of age compared to age-matched lean rats. The width of the glomerular basement membrane was significantly greater in *fa/fa* rats at 14 weeks of age compared to the lean rats. Hence, investigations

involving the obese *fa/fa* Zucker rat would give potential dietary approaches to modulate disease progression in OAN.

1.5 Hypotheses and Objectives

Therefore, we propose to use the obese *fa/fa* Zucker rat, an animal model of obesity to test the following hypotheses:

- Early dietary soy protein feeding will reduce deterioration in renal function and delay glomerular enlargement.
- Early dietary soy protein feeding will alter steady-state protein and mRNA levels of rate-limiting enzymes involved in prostanoid synthesis as well as the production of prostanoid themselves.

To test these hypotheses, 6-week-old obese male *fa/fa* Zucker rats and lean Zucker rats were given either 17% soy protein or egg white protein for 8 weeks.

The objectives were as follows:

- (1) To determine the effect of dietary soy protein on glomeruli size, by staining kidney sections with hemotoxylin and eosin, and to assess proteinuria and creatinine clearance as indicators of renal function.
- (2) To determine the effect of dietary soy protein on the steady-state levels of enzymes and gene expression in kidney tissue by western immunoblotting and real time RT-PCR.
- (3) To determine the effect of dietary soy protein on renal production of PGE₂, 6- keto prostaglandin F_{1α} (6-keto PGF_{1α}), stable metabolite of PGI₂, and

thromboxane B₂ (TXB₂), stable metabolite of TXA₂ by enzyme immunoassays.

2. Dietary Soy Protein Reduces Early Renal Disease Progression and Alters Prostanoid Production in Obese *fa/fa* Zucker Rats.

2.1 Abstract

Background. With the rising incidence of obesity and the metabolic syndrome, nephropathy associated with obesity also has increased. Soy protein can ameliorate disease in several models of chronic kidney disease. It also may alter the production of renal prostanoids, which play a role in the glomerular hyperfiltration in obesity-associated nephropathy (OAN). Therefore, the effect of dietary soy protein on early disease progression and prostanoid production in OAN was examined in the obese *fa/fa* Zucker rat.

Methods. Six-week-old male *fa/fa* and lean Zucker rats were offered complete diets *ad libitum* containing either 17% protein from soy protein or egg white protein for 8 weeks. Mean glomerular volume (MGV) and proteinuria were determined to assess early kidney changes and kidney function. Renal prostanoids [thromboxane B₂ (TXB₂), stable metabolite of TXA₂; 6-keto prostaglandin F_{1α} (6-keto PGF_{1α}), stable metabolite of PGI₂; and prostaglandin E₂ (PGE₂)] were determined by enzyme immunoassay. Renal protein and mRNA levels of cytosolic phospholipase A₂ (cPLA₂), cyclooxygenase-1 (COX-1), and COX-2 were determined by western immunoblotting and QRT-PCR.

Results. Feed consumption, body, and kidney weights were significantly greater in *fa/fa* compared to lean rats. The *fa/fa* rats had 43% larger MGV and 169% higher proteinuria, reflecting early development of nephropathy. Soy protein feeding did not alter body weights or proteinuria but did result in 6% lower kidney

weights (g/100 g body weight) and 16% lower MGV. Prostanoid production in these kidneys was primarily due to COX-2 activity. Renal prostanoids were generally not altered by diet or genotype, however, COX activity as determined by in vitro production of 6-keto PGF_{1α} was lower in *fa/fa* rats given soy compared to egg white protein based diets. Ratios of renal TXB₂/6-keto PGF_{1α} were higher and 6-keto PGF_{1α}/PGE₂ ratios were lower, while TXB₂/PGE₂ ratios were not different in rats given soy protein compared to egg white protein, also indicating that dietary soy protein reduces renal 6-keto PGF_{1α} levels.

Conclusion. The attenuation of early nephropathy in *fa/fa* rats by dietary soy protein is associated with lower 6-keto PGF_{1α} levels. This may affect the glomerular hyperfiltration observed in OAN.

Key Words: Dietary soy protein; Obesity-associated nephropathy; Cyclooxygenases; and Prostanoids

2.2 Introduction

Obesity has become an international epidemic. Even in children the incidence of obesity is rising with 10% of children world-wide now being considered obese (Baur, 2006). Obesity is associated with increased risk of a number of disorders including metabolic and cardiovascular diseases such as diabetes and hypertension. While both of these increase the risk for renal disease, obesity itself apart from these disorders also increases risk of renal disease (Srivastava, 2006).

Obesity increases glomerular filtration rate (GFR), apparently by due to dilation of the afferent arteriole while the efferent arteriole is not affected (Jong et al., 2002). Afferent arteriolar dilation leads to increased glomerular capillary pressure causing hyperfiltration, thickening of the glomerular basement membrane, and mesangial expansion that lead to glomerular enlargement. Functionally this early renal disease progression in obesity is also related to increased proteinuria. In the long term, these changes result in fibrosis in both the glomerulus and in the tubulointerstitial tissue (Jong et al., 2002; Phillips et al., 1999; Srivastava, 2006).

Prostanoids are known to be important regulators of renal hemodynamics and may be involved in early kidney changes that ultimately result in obesity-associated nephropathy (OAN). It is known that prostaglandin I₂ (PGI₂) and prostaglandin E₂ (PGE₂) have vasodilatory effects that increase GFR while thromboxane A₂ (TXA₂) has a vasoconstrictory effect that decreases GFR (Campean et al., 2003; Imig, 2000; Nasrallah et al., 2005; Smith & Murphy, 2002).

Individuals who have a BMI ≥ 25 (kg/m²) at age 20 have a 3-fold increased incidence of kidney failure (Ejerblad et al., 2006). This illustrates the impact of obesity in children on later health and incidence of renal disease. Therefore, early detection and treatment at the very beginning stages of renal disease associated with obesity are important.

Dietary protein level affects the progression of renal diseases, in part via alterations in prostanoid production (Stahl et al., 1987; Yamagisawa et al., 1994; Yamagisawa et al., 1998). The source of dietary protein also can affect disease progression. In this regard, dietary soy protein has been shown to benefit a number of renal diseases. Several of these studies indicate that renal hemodynamics are altered by soy or vegetable protein compared to animal protein sources, possibly by altering prostanoid production (Dhaene et al., 1987; Fair, 2001; Iwasaki et al., 1988; Kontessis et al., 1990; Peng, 2005). Therefore, the effect of dietary soy protein on early disease progression and prostanoid production in OAN was examined in the obese *fa/fa* Zucker rat.

2.3 Materials and Methods

2.3.1 Animals and Diet

Twenty lean and 20 obese *fa/fa* male Zucker rats were purchased from Harlan (Indianapolis, Indiana) at 5 wk of age, acclimated for 1 wk and then randomly divided into four groups in a 2 X 2 design. Ten lean and 10 *fa/fa* rats were given diets (**Table 4**) containing equal amounts of protein in the form of egg

Table 4. Diet formulation¹

Ingredients² (g/kg of diet)	Control Diet	Soy Protein Diet
Cornstarch ³	363.0	383.8
Maltodextrin	132.0	132.0
Sucrose	100.0	100.0
Egg white ⁴	212.5	0.00
Soy protein ⁵	0.00	197.7
Mineral Mix	35.0	35.0
Vitamin Mix	10.0	10.0
Choline	2.5	2.5
Biotin Mix ⁶	10.0	10.0
Tert-butylhydroquinone ⁷	0.014	0.014
Soy oil	85.0	79.0

¹ Diets were based on the AIN-93G diet.

² All ingredients supplied by Harlan Teklad (Madison, Wisconsin) unless otherwise indicated with

³ Best Foods (Etobicoke, On, Canada)

⁴ Egg white contains 80% protein

⁵ Soy protein contains 86% protein and 3 g oil/100 g isolate. Thus, less soy oil was added to soy protein diet.

⁶ biotin mix = 200 mg biotin/kg cornstarch

⁷ tert-butylhydroquinone supplied by Aldrich Chemical Company, Milwaukee, WI

white (17%) or soy protein (17%) as the control (CTL) or treatment diets, respectively. Body weights were recorded every week and feed intake was recorded three times per week. After 7.5 wk of the experimental diets, the rats were fasted overnight (12 hr) in metabolic cages to obtain urine. At 8 wks, rats were fasted overnight (12 hr) and killed the following morning by CO₂ gas asphyxiation and decapitation. Trunk blood was collected at this time for serum analysis. Serum and urine creatinine were measured colorimetrically using commercial kits (Sigma-Aldrich, Oakville, Canada) and the results were used to calculate creatinine clearance. Urine protein was determined using a colorimetric assay based on the method by Smith et al. (1985). This protein assay is based on the principle that protein will form a mixture with copper II which will then be reduced to copper I. The copper I will form a mixture with bicinchoninic acid (BCA) (Sigma, St. Louis, Missouri) in a concentration dependent manner. All procedures were approved by the University of Manitoba Animal Care Committee and the guidelines of the Canadian Council on Animal Care.

2.3.2 Glomerular Size

The right kidney was sliced longitudinally and half of the kidney was placed in 10% phosphate-buffered formalin prior to embedding in paraffin and sectioning at 5 microns. Kidney sections were placed in xylene to remove the paraffin and stained with hematoxylin and eosin. Using a camera (Spot Diagnostic Instruments, Inc., Sterling Heights, Michigan) mounted on an Olympus BX60 microscope (Olympus Optical Company, Hamburg, Germany), slides were

analyzed using the 20X objective. Section images containing at least 1 glomerulus were taken sequentially using the Spot Advanced software program. Thirty glomeruli were then measured using the Image Pro Plus 2 software program. The observer was blinded to treatments for all analyses.

2.3.3. Immunoblotting

Steady-state levels of cPLA₂, COX-1, and COX-2 protein expression were determined as described (Aukema et al., 2002). Half of the left kidney was lyophilized and 20 mg was homogenized in 100 volumes of ice-cold homogenization buffer (50 mM Tris-HCL, pH 7.2; 250 mM sucrose; 2 mM EDTA; 1 mM EGTA; 50 μ M NaF; 100 μ M Na orthovanadate; 1 μ g/ml soybean trypsin inhibitor; 144 μ M 4-benzene-sulfonyl fluoride; 25 μ g/mL aprotinin; 25 μ g/mL leupeptin; 25 μ g/mL pepstain; and 10 mM β -mercaptoethanol). Homogenates were centrifuged at 100,000 x g for 30 minutes at 4°C and the supernatant (cytosolic fraction) was removed. The remaining pellet was resuspended in 20 volumes of homogenization buffer containing 1% Triton X-100 (Sigma, St. Louis, Missouri), incubated on ice for 10 min and then centrifuged at 100,000 x g for 30 min at 4°C. The resulting supernatant (particulate fraction) was collected. Cytosolic and particulate fractions were subjected to SDS-PAGE as described.

After SDS-PAGE, proteins were transferred to PVDF, blocked, and incubated with primary antibodies to cPLA₂ (Santa Cruz Biotechnology Inc., Santa Cruz, CA), COX-1 and COX-2 (Cayman, Ann Arbor, Michigan) followed by incubation for 1 hr at room temperature with a peroxidase-conjugated secondary

antibody. Immunoblots were incubated with ChemiGlow™ (Alpha Innotech, San Leandro, California) and image analysis and quantitation of immunoreactive bands were performed using the Fluorchem™ FC digital imaging system (Alpha Innotech Corporation, San Leandro, California).

2.3.4 Prostanoid Production and COX Activity

Production of prostanoids and determination of COX isoform activities were analyzed as described (Warford-Woolgar et al., 2006). Briefly, lyophilized left kidneys from each rat were homogenized in fresh Tyrodes buffer and incubated under the following conditions as described: (1) 0 min with no inhibitor for determination of endogenous levels of prostanoid production; (2) 60 min incubation at 37°C with no inhibitor for determination of steady-state in vitro prostanoid production; (3) 10 min incubation at 37°C with no inhibitor for determination of total COX activity of prostanoid production; (4) 10 min incubation at 37°C with 0.1 µM SC560 (Cayman, Ann Arbor, Michigan) for determination of COX-2 activity of prostanoid production. COX-1 activity was determined by the difference between total COX (condition 3) and COX-2 (condition 4) activities.

The incubation conditions were determined from previous time course studies which demonstrated that the production of prostanoids is linear for the first 10 min of incubation, that steady-state levels of prostanoids are achieved by 30-40 min of incubation, and that a concentration of 0.1 µM SC560 inhibits more than 90% of COX-1 activity but does not inhibit COX-2 at all (Warford-Woolgar et

al., 2006). Reactions were stopped by adding cold ASA to the sample incubation, vortexing and centrifuging at 12,000 x g at 4°C for 5 min. The supernatant was removed for determination of PGE₂, 6-keto PGF_{1α}, (stable metabolite of PGI₂), and TXB₂, (stable metabolite of TXA₂), using commercial enzyme immunoassay kits (Cayman, Ann Arbor, Michigan).

2.3.5 Quantitative RT-PCR

Total RNA for real time RT-PCR was extracted from 20 mg of lyophilized kidney. Primers for RT-PCR were chosen using Primer 3 software (Rozen & Skaletsky, 2003). Oligonucleotide sequences were as follows: sense cPLA₂ primers were 5'-GACTTTTCTGCAAGGCCAAG-3' and antisense 5'-CTTCAATCCTTCCCGATCAA-3', COX-1 sense were 5'-GCCTCGACCACTACCAATGT-3' and antisense 5'-AGGTGGCATTACAAACTCC-3', COX-2 sense were 5'-TACCCGGACTGGATTCTACG-3' and antisense 5'-TTCGAAGGAAGGGAATGTTG-3'. Real time RT-PCR reactions were performed with SYBR green on a Cepheid Smart Cycler II (Cepheid, Sunnyvale, CA) sequence detection system. Products were verified by melting curve analysis. Relative amounts of mRNA were determined by comparing cycle threshold (CT) values for equal amounts of amplified RNA and calculated using the formula $2^{\Delta CT}$ as described (Warford-Woolgar et al., 2006).

2.3.6 Statistical Analysis

Data were analyzed for normality using the Shapiro-Wilk Statistic and for homogeneity of variance using Levene's Test for Homogeneity of Variance. Data that was not normally distributed was log transformed. A two-way analysis of variance (ANOVA) was used to analyze main [diet (CTL/soy), genotype (lean/*fa/fa*)] effects and interactions. If interactions were present ($P < 0.05$) or when the P-value of main effects were marginal ($0.05 < P < 0.10$), least significant difference (LSD) tests were performed to test for differences among groups. Data were analyzed using SAS (SAS Institute, version 9.1, Cary, North Carolina).

2.4 Results

At the end of the feeding period, *fa/fa* rats had consumed approximately 50% more diet and were significantly larger than lean rats (**Table 5**). Rats grew equally well on both diets, despite the slightly lower feed intake (7%) in *fa/fa* rats given the soy based compared to the CTL diet. The *fa/fa* rats had larger kidneys than lean rats at the end of the study on a weight basis, but relative to body weights, the kidney weights in *fa/fa* rats were smaller than in lean rats. There was a significant effect of dietary soy protein with rats given soy protein having lower kidney weights relative to body weights compared to CTL diets.

Table 5. Effects of dietary soy protein on feed intake, body and kidney weights, proteinuria, and creatinine clearance in obese *fa/fa* Zucker rats. Values are mean \pm SEM. Different letters in rows are significantly different, $P < 0.05$.

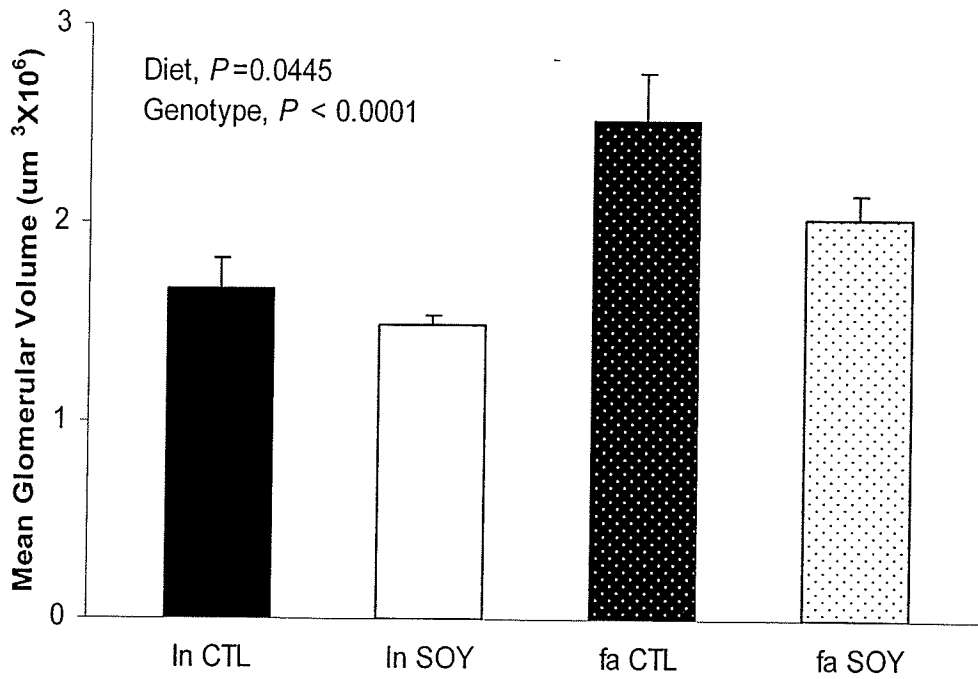
Genotype Diet	Lean		<i>fa/fa</i>		Main Effects
	Control	Soy	Control	Soy	
Total feed intake, g	1022 $\pm 24^c$	1101 $\pm 15^c$	1666 $\pm 51^a$	1551 $\pm 36^b$	Interaction ($P=0.0074$)
Body weight, g	328.3 ± 5.3	348.9 ± 8.5	561 ± 12.6	568.1 ± 8.0	Genotype ($P<0.0001$)
Kidney weight, g	2.33 ± 0.08	2.34 ± 0.05	3.03 ± 0.09	2.83 ± 0.05	Genotype ($P<0.0001$)
Kidney weight, g/ 100g body weight	0.71 ± 0.02	0.67 ± 0.01	0.54 ± 0.01	0.50 ± 0.01	Diet ($P=0.0106$) Genotype ($P<0.0001$)
Urinary protein, mg /Creatinine, mg	1.37 ± 0.24	1.77 ± 0.21	3.01 ± 0.40	4.48 ± 0.69	Genotype ($P<0.0001$)
Creatinine Clearance, mL/min	1.47 ± 0.16	1.63 ± 0.14	1.41 ± 0.22	1.17 ± 0.10	No effect

One of the earliest signs of OAN is an increase in glomerular size. Glomerular size was elevated in *fa/fa* rats as indicated by the 43% larger mean glomerular volume (MGV) in these rats compared to lean rats (**Figure 1 and 2**). This increased size was mitigated by soy protein feeding which resulted in 16% lower MGVs compared to the CTL fed rats. These early kidney changes reflecting renal damage in *fa/fa* rats were reflected in the increased proteinuria in these rats, but serum creatinine and creatinine clearance were not altered in this early stage of OAN (**Appendix 7.5**). The dietary protein source did not influence these markers of renal function.

To determine the potential role of prostanoids in these effects, prostanoid levels and COX activities were determined. These analyses determined that renal COX activity was due primarily to the COX-2 isoform as can be seen in the similar levels of total COX and COX-2 activities (**Tables 6-9**). Renal prostanoid levels and COX activities were generally not altered by diet or genotype. However, renal 6-keto PGF_{1α} levels produced by COX activity were lower in *fa/fa* rats fed soy protein, which suggested that dietary soy protein reduces 6-keto PGF_{1α} production as result of COX activity.

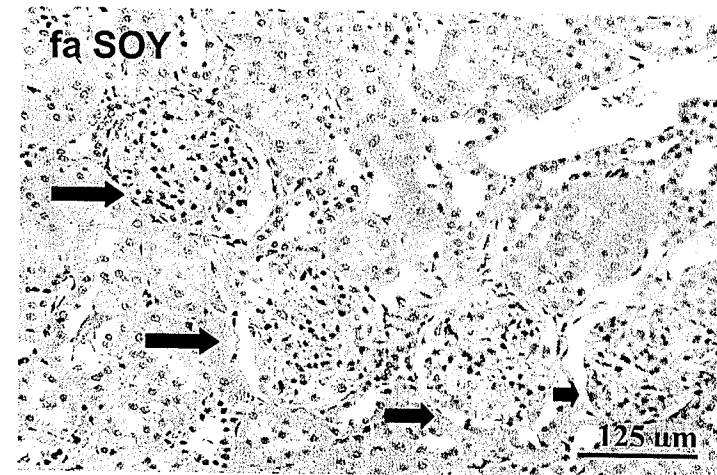
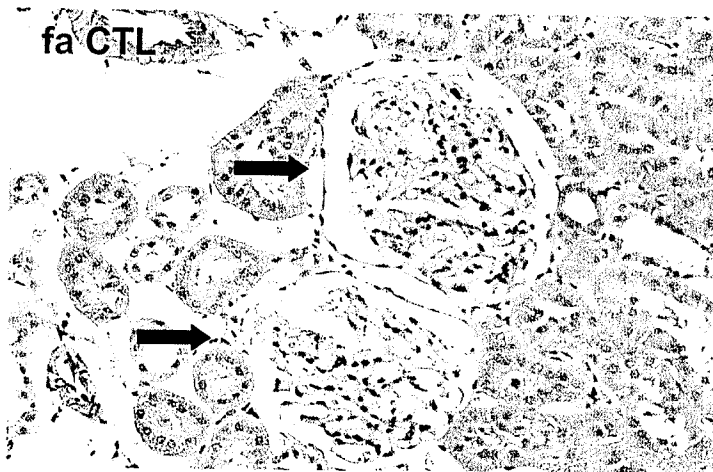
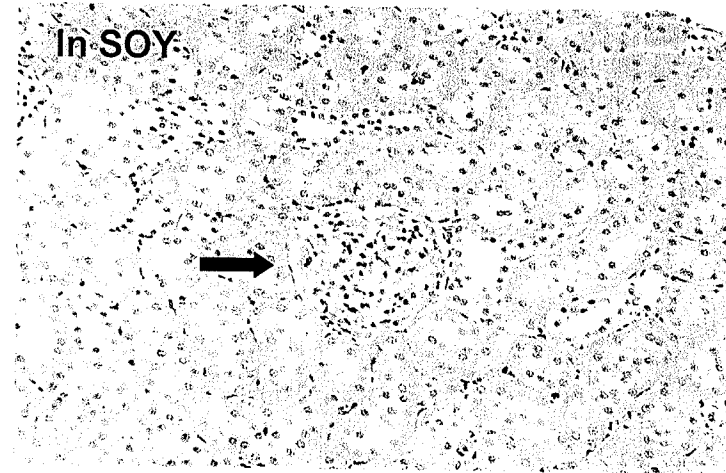
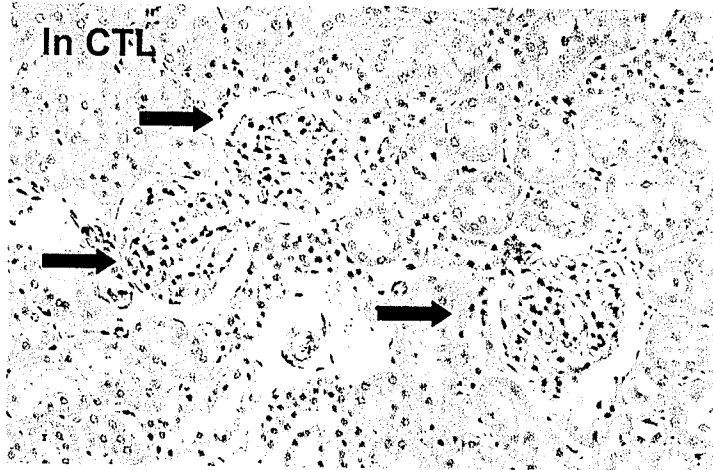
In order to probe further the possible effects of dietary soy protein on 6-keto PGF_{1α}, ratios were calculated and compared to determine whether there were changes in individual prostanoids relative to others. Renal TXB₂/6-keto PGF_{1α} ratios were significantly higher in the kidneys from rats given soy protein

Figure 1. Effects of dietary soy protein on mean glomerular volume (MGV). Values are mean \pm SEM (n=10/group, except n=9 for In SOY)¹.



¹ Abbreviations: In CTL, lean rats fed control diet; In SOY, lean rats fed soy protein diet; fa CTL, *fa/fa* rats fed control diet; and fa SOY, *fa/fa* rats fed soy protein diet

Figure 2. Kidney cross sections stained with eosin and hematoxylin¹. (Arrows point to glomeruli.)



¹ Abbreviations: In CTL, lean rats fed control diet; In SOY, lean rats fed soy protein diet; fa CTL, *fa/fa* rats fed control diet; and fa SOY, *fa/fa* rats fed soy protein diet

Table 6. Effects of dietary soy protein on endogenous prostanoid levels (ng/mg protein) in kidneys of obese *fa/fa* Zucker rats. Values are mean \pm SEM (n=9-10/group).

Genotype	Lean		<i>fa/fa</i>		Main Effects
	Control	Soy	Control	Soy	
TXB ₂	0.15 \pm 0.02	0.16 \pm 0.02	0.14 \pm 0.02	0.14 \pm 0.02	No effect
PGE ₂	1.07 \pm 0.20	1.07 \pm 0.19	0.58 \pm 0.08	0.71 \pm 0.10	Genotype (<i>P</i> =0.0068)
6-keto PGF _{1α}	1.86 \pm 0.26	1.77 \pm 0.28	1.77 \pm 0.28	1.18 \pm 0.14	No effect
Total	3.01 \pm 0.44	2.90 \pm 0.46	2.18 \pm 0.32	2.00 \pm 0.26	Genotype (<i>P</i> =0.0368)

Table 7. Effects of dietary soy protein on in vitro steady-state prostanoid levels (ng/mg protein) in kidneys of obese *fa/fa* Zucker rats. Values are mean \pm SEM (n=9-10/group).

Genotype	Lean		<i>fa/fa</i>		Main Effects
	Control	Soy	Control	Soy	
TXB ₂	0.16 \pm 0.02	0.18 \pm 0.03	0.24 \pm 0.04	0.21 \pm 0.03	No effect
PGE ₂	2.97 \pm 0.34	3.31 \pm 0.54	2.72 \pm 0.45	2.94 \pm 0.40	No effect
6-keto PGF _{1α}	13.97 \pm 1.65	15.37 \pm 2.96	20.86 \pm 3.54	13.98 \pm 1.70	No effect
Total	17.00 \pm 1.89	18.69 \pm 3.50	20.18 \pm 2.07	17.13 \pm 2.12	No effect

Table 8. Effects of dietary soy protein on total COX activity (ng/min/mg protein) in kidneys of obese *fa/fa* Zucker rats. Values are mean \pm SEM (n=9-10/group).

Genotype	Lean		<i>fa/fa</i>		Main Effects
	Control	Soy	Control	Soy	
TXB ₂	0.01 \pm 0.00	0.01 \pm 0.00	0.02 \pm 0.00	0.02 \pm 0.00	No effect
PGE ₂	0.20 \pm 0.02	0.25 \pm 0.05	0.22 \pm 0.04	0.24 \pm 0.04	No effect
6-keto PGF _{1α}	0.86 \pm 0.14 ^{ac}	0.83 \pm 0.17 ^{bc}	1.24 \pm 0.2 ^a	0.77 \pm 0.10 ^{bc}	Diet (<i>P</i> =0.0628)
Total	1.07 \pm 0.15	1.08 \pm 0.22	1.27 \pm 0.16	1.01 \pm 0.15	No effect

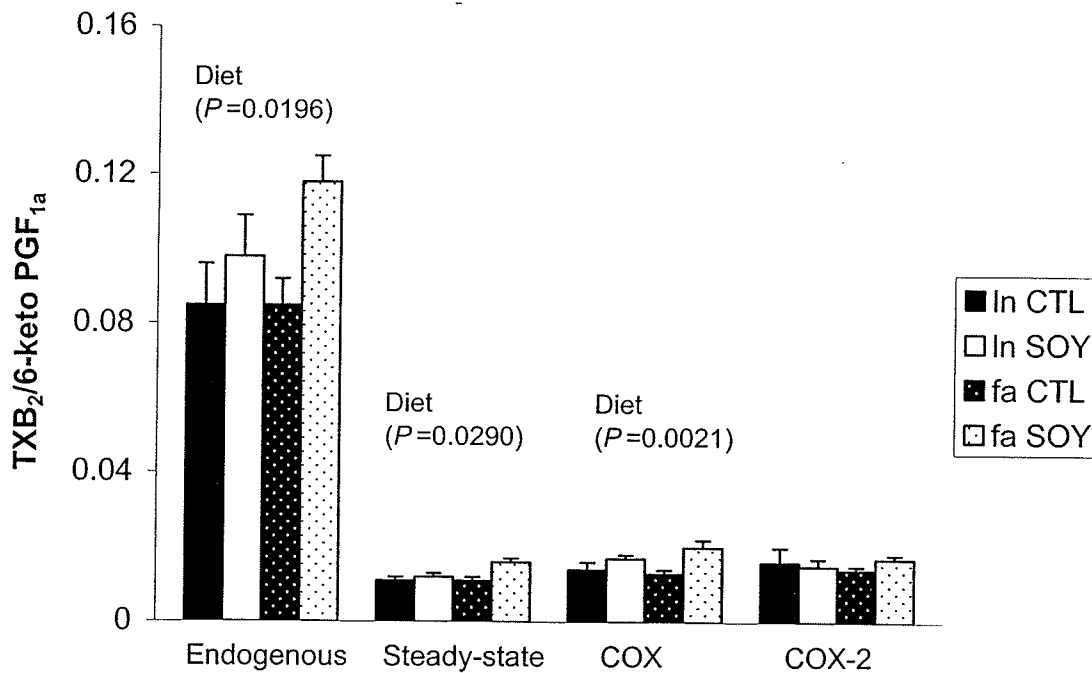
Table 9. Effects of dietary soy protein on COX-2 activity (ng/min/mg protein) in kidneys of obese *fa/fa* Zucker rats. Values are mean \pm SEM (n=9-10/group).

Genotype	Lean		<i>fa/fa</i>		Main Effects
	Control	Soy	Control	Soy	
TXB ₂	0.01 \pm 0.00	0.01 \pm 0.00	0.02 \pm 0.00	0.02 \pm 0.00	No effect
PGE ₂	0.19 \pm 0.02	0.24 \pm 0.04	0.22 \pm 0.04	0.21 \pm 0.03	No effect
6-keto PGF _{1α}	0.86 \pm 0.13	0.88 \pm 0.15	1.17 \pm 0.16	0.88 \pm 0.11	No effect
Total	1.07 \pm 0.15	1.12 \pm 0.19	1.25 \pm 0.16	1.10 \pm 0.13	No effect

compared to CTL diets indicating that dietary soy protein reduced either 6-keto $\text{PGF}_{1\alpha}$ and/or increased TXB_2 (**Figure 3**). Similarly, 6-keto $\text{PGF}_{1\alpha}/\text{PGE}_2$ ratios were lower in soy protein fed rats compared to the CTL diet, indicating that dietary soy protein either reduced renal 6-keto $\text{PGF}_{1\alpha}$ levels and/or increased PGE_2 (**Figure 4**). Renal $\text{TXB}_2/\text{PGE}_2$ ratios, on the other hand, were not different (**Figure 5**). It is not likely that these prostanoid levels are altered, as the individual prostanoid measurements do not support that interpretation. Therefore, taken together, these results suggest that 6-keto $\text{PGF}_{1\alpha}$ is reduced while PGE_2 and TXB_2 are not altered.

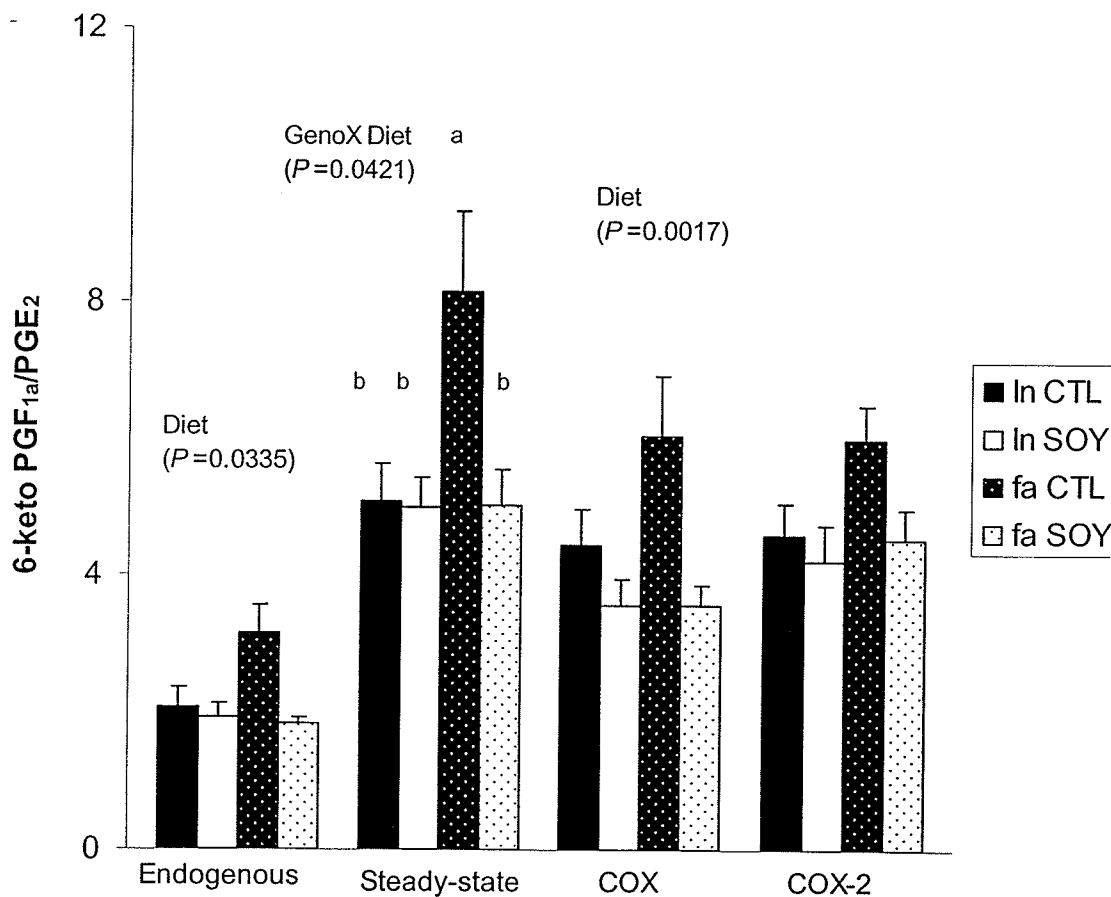
COX-2 protein and mRNA expression were elevated in kidneys from *fa/fa* compared to lean rats, as determined by western immunoblotting (**Figure 6**) and QRT-PCR (**Figure 7**). Dietary soy protein did not alter the expression of COX-2 protein, though gene expression was altered by diet. The COX isoforms were present only in the particulate fraction, while cPLA₂ was present in both the cytosolic and particulate fractions. Neither COX-1 nor cPLA₂ levels were altered by genotype or diet.

Figure 3. Effects of dietary soy protein on TXB₂/6-keto PGF_{1α} ratio in kidneys of obese *fa/fa* Zucker rats in the following conditions: 0-min incubation (endogenous levels), 60-min incubation (in vitro steady-state levels), COX activity, and COX-2 activity. Values are mean ± SEM (n=8-10/group)¹.



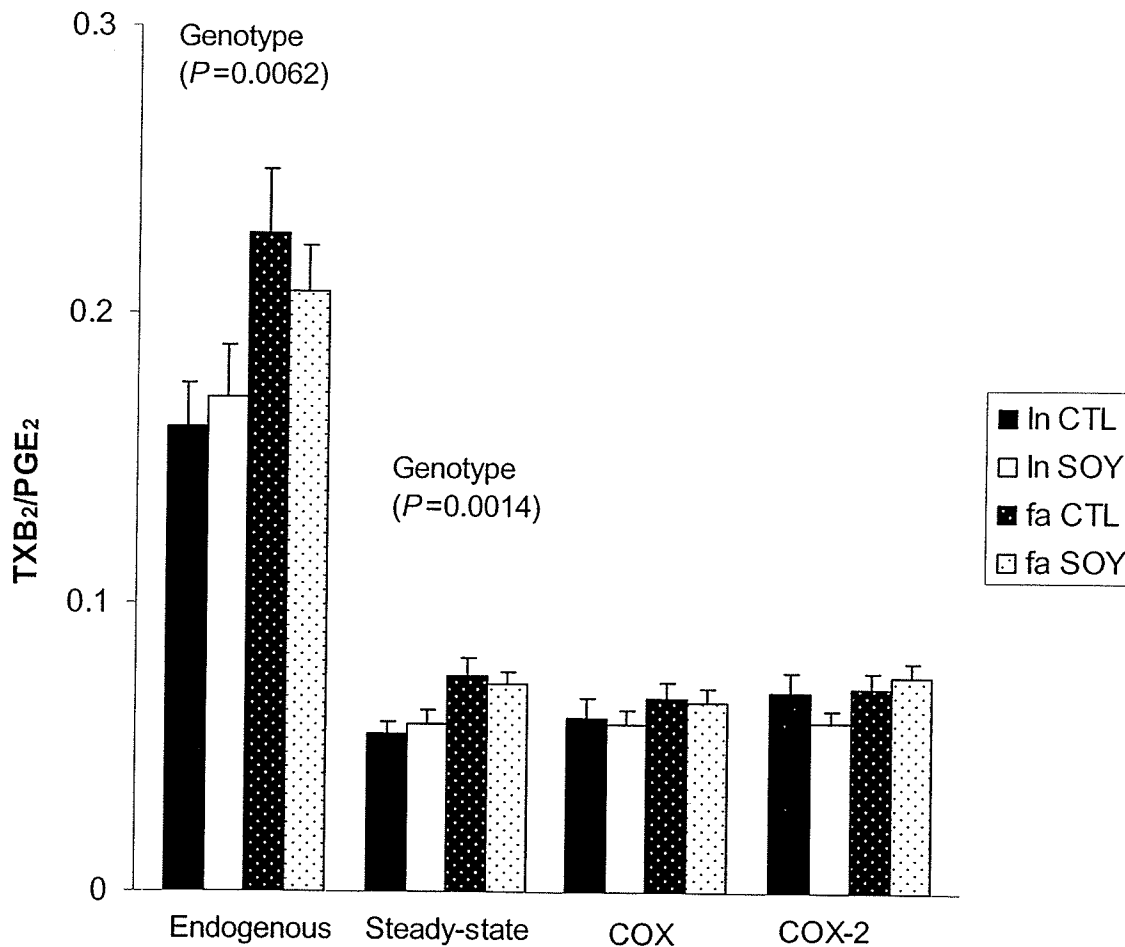
¹ Abbreviations: COX, cyclooxygenase; COX-2, cyclooxygenase-2; In CTL, lean rats fed control diet; In SOY, lean rats fed soy protein diet; fa CTL, *fa/fa* rats fed control diet; and fa SOY, *fa/fa* rats fed soy protein diet

Figure 4. Effects of dietary soy protein on 6-keto PGF_{1α}/ PGE₂ ratio in kidneys of obese *fa/fa* Zucker rats in the following conditions: 0-min incubation (endogenous levels), 60-min incubation (in vitro steady-state levels), COX activity, and COX-2 activity. Values are mean ± SEM (n=8-10/group)¹. Different letters in columns are significantly different, *P* < 0.05.



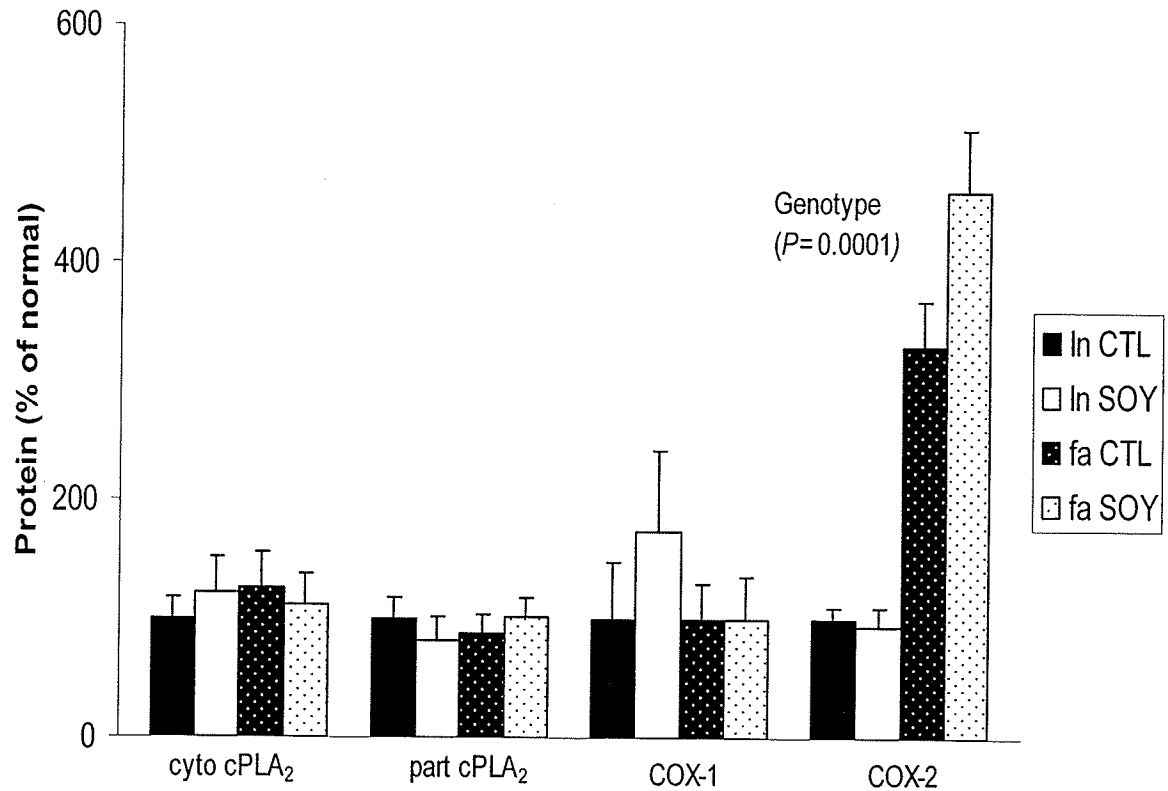
¹ Abbreviations: COX, cyclooxygenase; COX-2, cyclooxygenase-2; In CTL, lean rats fed control diet; In SOY, lean rats fed soy protein diet; fa CTL, *fa/fa* rats fed control diet; and fa SOY, *fa/fa* rats fed soy protein diet

Figure 5. Effects of dietary soy protein on TXB₂/PGE₂ ratio in kidneys of obese *fa/fa* Zucker rats in the following conditions: 0-min incubation (endogenous levels), 60-min incubation (in vitro steady-state levels), COX activity, and COX-2 activity. Values are mean ± SEM (n=8-10/group)¹.



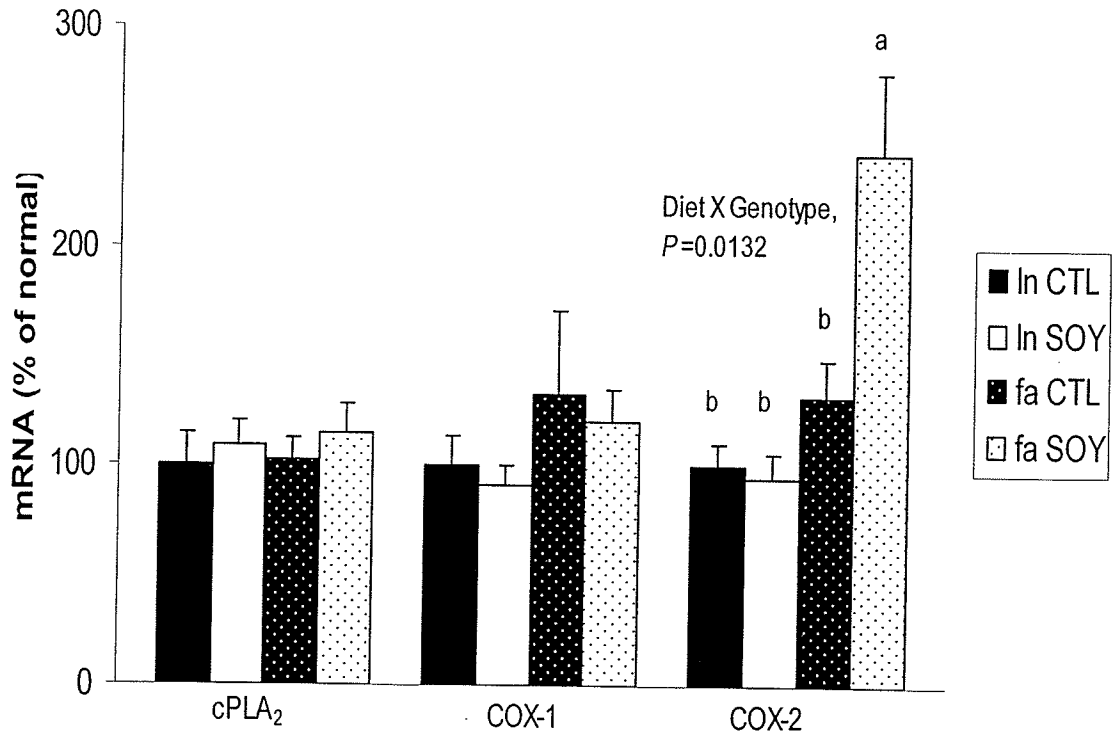
¹ Abbreviations: COX, cyclooxygenase; COX-2, cyclooxygenase-2; In CTL, lean rats fed control diet; In SOY, lean rats fed soy protein diet; fa CTL, *fa/fa* rats fed control diet; and fa SOY, *fa/fa* rats fed soy protein diet

Figure 6. Effects of dietary soy protein on relative cytosolic cPLA₂, particulate cPLA₂, COX-1, and COX-2 protein expression¹. Data are expressed relative to In CTL group and as mean ± SEM (n=8-10/group).



¹ Abbreviations: cyto cPLA₂, cytosolic fraction of cytosolic phospholipase A₂; part cPLA₂, particulate fraction of cytosolic phospholipase A₂; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; In CTL, lean rats fed control diet; In SOY, lean rats fed soy protein diet; fa CTL, *fa/fa* rats fed control diet; and fa SOY, *fa/fa* rats fed soy protein diet

Figure 7. Effects of dietary soy protein on relative cPLA₂, COX-1, and COX-2 mRNA expression. Data are expressed relative to In CTL group and as mean \pm SEM (n=8-10/group)¹. Different letters in columns are significantly different, $P < 0.05$.



¹ Abbreviations: cPLA₂, cytosolic phospholipase A₂; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; In CTL, lean rats fed control diet; In SOY, lean rats fed soy protein diet; fa CTL, *fa/fa* rats fed control diet; and fa SOY, *fa/fa* rats fed soy protein diet

2.5 Discussion

This study confirms the beneficial effect of dietary soy protein intervention on early renal disease progression associated with obesity in the obese *fa/fa* Zucker rat. The soy protein diet provides protection against the progression of glomerular injury, as demonstrated by reduced MGV.

Glomerular hypertrophy is one of the earliest sign of kidney disease and has been proposed as an independent risk factor for the progression of renal disease. The beneficial effect of soy protein on glomerular size observed herein in 14 wk old rats is consistent with previous observation in 24 wk obese *fa/fa* Zucker rats (Maddox et al., 2002). One difference between the previous study and the present study is the rats from the previous study were older, therefore, and kidney disease was already established. Another difference is the previous study used casein as an animal protein source versus soy protein. In this current study we used egg white protein which is a high quality animal protein. The lack of significant effects on renal function is likely due to the very early stage of renal disease in the 14 wk Zucker rat.

OAN is characterized by glomerular hyperfiltration, increased glomerular pressure and size, and subsequent development of fibrosis (Jong et al., 2002). The current findings suggest that the effect of dietary soy protein on renal 6-keto $\text{PGF}_{1\alpha}$ may be a mechanism by which soy protein mediates its beneficial effect on glomerular size.

How these prostanoids are influenced by soy protein may be analogous to the effects of high protein diets that have been shown to alter prostaglandins in

kidneys and lead to hyperfiltration. Low compared to high protein diets ameliorate these changes in prostaglandin production and hyperfiltration and renal disease progression (Stahl et al., 1987; Yamagisawa et al., 1994; Yamagisawa et al., 1998).

Altering the protein source is another approach that has been used in treating renal disease. It is known that plant compared to animal proteins alter renal hemodynamics (Dhaene et al., 1987; Iwasaki et al., 1988; Kontessis et al., 1990). One such protein source that has been studied in several models of renal disease is soy protein. Studies from our laboratory have demonstrated that soy protein can alter prostanoid production in rats with renal cyst disease (Fair et al., 2004; Peng, 2005). In this present study, dietary soy protein reduces the production of 6-keto PGF_{1α}. The reduction in 6-keto PGF_{1α} production would reduce the dilation of the afferent arteriole and the glomerular capillary pressure which causes glomerular hyperfiltration.

Our previous study showed that dietary soy protein attenuated the higher protein levels of particulate cPLA₂ in the Han:SPRD-cy rat, a rat model of polycystic kidney disease (Peng, 2005). In another study, COX-2 protein expression was significantly reduced in the obese *fa/fa* Zucker rat, the same rat model as in this current study, when given dietary conjugated linoleic acid (CLA) (Herchak, 2005). In the present study, there were no significant diet or genotype effects for cPLA₂ protein and mRNA expression. In contrast, COX-2 protein expression was elevated in *fa/fa* rats, and this result is consistent with our previous studies and others (Dey et al., 2004; Herchak et al., 2005; Komers et al.,

2005; Warford, 2003; Xu et al., 2005). Higher levels of this enzyme appear to be related to renal disease progression. In addition, Xu et al. show higher COX-2 protein and mRNA expression in the kidney cortex of *fa/fa* Zucker rats compared to lean rats (Xu et al., 2005). Interestingly, while there was no significant dietary effect on protein expression, in this present study COX-2 mRNA expression in kidneys from *fa/fa* rats given soy protein was elevated compared to CTL diet. There were no significant effects for COX-1 protein and mRNA expression in this current study, which agrees with the results of our previous studies in the same rat model (Herchak, 2005; Warford, 2003).

In conclusion, the present study indicates that dietary soy protein attenuates early renal development of nephropathy in the obese *fa/fa* Zucker rats. These effects are associated with lower 6-keto PGF_{1α} levels. With obesity, glomerular size increases due to dilation of the afferent arteriole. In this present study, when soy protein is given in the diet, the reduction in 6-keto PGF_{1α} production would reduce this dilation and the glomerular capillary pressure. The prevalence of the metabolic syndrome in U.S. is greater than 47 million with the rising incidence of obesity (Abrass, 2004; Bagby, 2004; Tuttle, 2005). An aspect of metabolic syndrome associated with chronic renal disease suggests that people with obesity are at increased risk for progressive loss of renal function. More importantly, obesity is not only a problem for the adult but is becoming a concern for the young population (Csernus et al., 2005). Hence, an early intervention strategy is important to prevent and/or slow early OAN.

3. Overall Discussion

The epidemic of obesity in adult, as well as in both boys and girls, is rapidly increasing throughout the world. This trend mirrors a concurrent rise in the prevalence of related chronic diseases including kidney disease internationally (Lobstein et al., 2004). In addition, obesity worsens disease in individuals with underlying kidney disease.

Some strategies to slow renal disease progression include treatment of blood pressure using angiotensin-converting enzyme inhibitors and angiotensin receptor blockers and dietary intervention (Hostetter, 2003). Early studies showed that dietary protein restriction modified the course of progressive renal disease (Brenner et al., 1982; Kasiske et al., 1998; Klahr & Purkerson, 1998; Williams et al., 1987; Zeller et al., 1991). However, the use of dietary protein restriction is controversial because of the risk of protein malnutrition and the difficulty of maintaining this diet in the long-term. Altering the source of protein may be beneficial in delaying renal disease progression.

Previous studies report that consumption of plant-derived proteins such as soy protein retards the progress of renal disease in several animal models and, in humans improves renal function and reduces renal damage (Anderson et al., 1998; Maddox et al., 2002; Ogborn et al., 1998; Teixeira et al., 2003; Tovar et al., 2002; Trugillo et al., 2005; Williams et al., 1987). In addition, recent studies show that soy protein delays progression of renal disease in the early stages, again suggesting the beneficial effects of soy protein (Fair et al., 2004; Peng, 2005).

Kidneys are a relatively rich source of prostanoids, which are important regulators of kidney physiology in health and in disease. In normal kidneys prostanoids regulate renal processes such as hemodynamics, water and solute transport and renin secretion. Prostanoids in diseased kidneys play a role maintaining glomerular filtration rate (GFR) as well as being involved in proliferative and inflammatory processes in response to renal injury.

The present study demonstrates the effects of dietary soy protein intervention on early renal disease progression in obesity. Rats given soy had lower kidney weights (g/100 g body weight) and lower MGW compared to rats fed the CTL diet. Feed consumption, body weights, and kidney weights were significantly greater in *fa/fa* compared to lean rats. Since the *fa/fa* rat model is hyperphagic, a greater total feed intake is to be expected. The hyperphagia observed in this rat model is a result of an inactivating mutation in the leptin receptor gene. Leptin is a hormone responsible for maintaining a balance between energy expenditure and food intake (Bray et al., 1977). Since leptin does not function properly in this rat model, *fa/fa* rats are unable to suppress their appetites. In this study, *fa/fa* rats fed soy protein had a 41% greater feed intake than lean rats fed soy. In addition, *fa/fa* rats fed CTL had a 63% greater feed intake compared to lean rats fed CTL. However, *fa/fa* rats fed soy protein had a 7% smaller feed intake compared to *fa/fa* rats fed CTL.

The *fa/fa* rats, as their name implies, were 67% heavier than lean rats. However, the final body weight was not significantly altered by diet. This raises the question of why a reduced feed intake did not lead to a reduced body weight.

It might be because the obese *fa/fa* Zucker rats are more efficient in converting their feed to weight gain than lean rats, as reported by Sisk et al. (2001). In their study, 0.5% dietary CLA reduced adiposity in the lean rats but not the *fa/fa* rats. Bray et al. also reported that pair feeding of *fa/fa* Zucker rats to the feed intake of lean rats did not lead to the normalization of body fat content nor to the elimination of the characteristic defects of these rats (Bray, 1977). These studies support the finding that reduced feed intake in soy protein fed obese *fa/fa* Zucker rats did not affect their final body weight.

Urine protein to urine creatinine is expressed to overcome the possible misinterpretation of higher total urinary protein excretion because of higher total volume seen in *fa/fa* rats. The *fa/fa* rats had a 169% higher urinary protein/creatinine ratio compared to the lean rats. However, there were no significant effects of diet shown in this ratio. Twelve-hour total urinary creatinine excretion was lower in *fa/fa* rats compared to lean rats; CTL fed rats had significantly lower (12 hr) urine creatinine than soy fed rats (**Appendix 7.5**). The *fa/fa* rats are known to have an altered renal structure and impaired renal function, as evidenced by larger glomerular area and proteinuria, respectively (Bray, 1977; Janssen et al., 1999). Since creatinine cannot be properly excreted in the urine due to impaired renal function in *fa/fa* rats compared to lean rats, one might expect it to build up in the serum. Interestingly, however, no significant effects were found in serum creatinine, creatinine clearance, or creatinine clearance relative to 100 g body weight (**Appendix 7.5**).

Since a higher MGV is indicative of impaired renal function in the *fa/fa* rat model, attenuating this enlargement by soy protein would likely be beneficial. Glomerular hypertrophy, which has been proposed as an independent risk factor for the progression of renal disease and has been observed in the obese Zucker rat as evidenced by larger MGV in the *fa/fa* rats compared to lean rats, agrees with our previous studies and other studies (Herchak, 2005; Kasiske et al, 1985; Maddox et al., 202; Warford, 2003). In the present study, the soy protein diet provides protection against the progression of glomerular injury, as demonstrated by reduced MGV.

In this study, there were no significant diet or genotype effects for cytosolic and particulate cPLA₂. Our previous study showed that dietary soy protein attenuated the higher protein levels of particulate cPLA₂ in the Han:SPRD-*cy* rat (Peng, 2005). Cytosolic cPLA₂ protein expression was higher in the obese *fa/fa* Zucker rat, the same rat model as in the current study (Herchak, 2005). The enzyme cPLA₂ is known to be responsible for the first step of prostanoid synthesis by releasing arachidonic acid (AA) from membrane phospholipids. Cytosolic cPLA₂ is an inactive form and is stimulated by cytosolic-free calcium, then becomes activated by binding to cellular membranes (Evans et al., 2001; Hirabayashi & Shimizu, 2001; Kramer & Sharp, 1997).

No significant effects were observed for this protein level in this study with in the same rat model but particulate cPLA₂ was higher in the *fa/fa* rats compared to lean rats (Warford, 2003). This might be because of the different diet effects or different progression of renal disease. Even though dietary soy protein

attenuated the higher protein levels of particulate cPLA₂ in rats with renal cyst disease, the animal model is different and with that model dietary soy protein did not alter cPLA₂ in the cytosolic fraction or COX-1 and COX-2 enzyme levels.

In this current study, renal prostanoids were generally not altered by diet; there were few genotype effects on the endogenous or in vitro synthesized levels, and diet had no effect on these prostanoid levels, however, renal 6-keto PGF_{1α} levels were lower in rats fed soy protein. One of our previous studies showed that dietary soy protein feeding lowered the in vitro steady-state levels of TXB₂ and attenuated COX activity that produced TXB₂ (Peng, 2005). Increased PGE₂ levels in diseased kidneys by dietary soy protein was found in weanling Han:SPRD-cy rats at 1 and 3 wk compared to control diet (Fair et al., 2004).

Studies have revealed that soy protein intake ameliorates progression of renal disease. In db/db mice, which are a model of type 2 diabetes and diabetic nephropathy, dietary soy protein slows development of diabetic nephropathy (Teixerira et al., 2003). It also reduces renal cysts and renal fibrosis in animal models of renal cyst disease (Ogborn et al., 1998; Ogborn et al., 2000; Tomobe et al., 1998). Even in aging Fischer 344 rats dietary soy protein feeding increases life span and decreases renal pathology (Iwasaki et al., 1988; Sisk et al., 2001). In the obese *fa/fa* Zucker rat model, which is the same rat model used in our study except that the rats were older (24 wk) and kidney disease was established, a soy protein diet (23%) slows the development of proteinuria and glomerular injury in adult rats (Maddox et al., 2002).

Even though the exact mechanism of the renal soy protective effect is not established, specific components in soy protein compared with egg white protein may be responsible for these dietary effects.

Isoflavones, a component in soy protein, have been shown to possess anti-proliferative and anti-inflammatory properties. It has been reported that genistein can inhibit protein tyrosine kinase that is associated with cellular receptors for growth factors and transcription factors involved in cell inflammation and oxidative stress (Akiyama et al., 1987; Setchell, 1998). Renal protective effects of isoflavones also could result from anti-oxidant properties, which could not only interfere with formation of free radicals, but also result in enhancing nitric oxide availability, an important factor in defining the progression rate of renal disease.

Several studies have reported that nitric oxide synthesis is reduced in both humans and animals with renal disease suggesting that an impaired nitric oxide synthetic pathway may have an important role associated with the progression of renal disease (Schmidt and Baylis, 2000; Wagner et al., 2002). Recently Trugillo et al. demonstrated that dietary soy protein improved urinary nitrites and nitrates excretion compared to dietary casein in obese Zucker rats. This result is associated with improvement of renal function evidenced by less proteinuria, glomerulosclerosis, tubular dilation, and interstitial fibrosis (Trugillo et al., 2005).

In conclusion, our present study indicates that dietary soy protein attenuates early renal development showing smaller MGW and kidney weight relative to body weight in the *fa/fa* rat model of OAN. This protective effect of soy protein may be related to specific effects on renal 6-keto PGF_{1 α} prostanoid production. It

is reported that approximately 9 million American children over 6 years of age are considered obese (Lobstein et al., 2004). This leads to a concern regarding the potential impact of obesity in children on later health related to kidney disease. Therefore, an early intervention to prevent and/or delay OAN would have significant benefits.

4. Strengths and Limitations

A strong point of this study is the animal model used. The male obese *fa/fa* Zucker rat is a good model of obesity and metabolic syndrome. Therefore, investigations involving dietary intervention during this disease state may aid in early amelioration of renal disease progression and/or even the prevention of renal disease.

Another strength is that dietary intervention began at an early stage in disease progression. It is important to focus on prevention strategies of kidney disease before the disease has significantly progressed. Therefore, the beneficial effects observed in this study may provide a potential approach to have more impact on renal disease progression when dietary soy protein intervention is started earlier.

Another strength of this study is that, to the writer's knowledge, it is the first study investigating the effect of dietary soy protein intervention to determine prostanoid production and prostanoid enzyme activities in the obese *fa/fa* Zucker rat model.

Several limitations exist in this present study. One limitation of this study is that this study was done using a rat model instead of humans. Even though the animal model used in this current study is a good model to study OAN since this rat model closely resembles human obesity, a human trial is necessary to compare what we have found with respect to beneficial effects of dietary soy protein. Another limitation of this study is that all parameters investigated were done only at the end of study. Therefore, conclusions regarding changes of the disease progression could not be stated.

Another limitation is that body composition was not analyzed in these rats, which made it difficult to interpretate the data with kidney weights expressed by body weights.

Another limitation of this study is that the soy protein we used is not a pure protein. Therefore, we are not able to determine if the beneficial effects of soy protein intervention observed in our study are caused by biologically active compounds within the soy protein isolate or by soy protein itself.

5. Future Research

For further investigations of dietary soy protein in OAN, there are several potential directions to follow:

- Measure individual renal prostanoid production and COX isoforms in different renal cell types. Renal prostanoids and COX isoforms are not present homogenously in kidneys; therefore, detecting their specific distribution and their

changes by dietary soy protein intervention would help to establish the mechanisms of beneficial effects of soy protein on OAN.

- Try different amounts of soy protein. In the current study, only one level of soy protein (20%) was used. Therefore, studies examining whether or not higher amounts of soy protein would result in greater reductions in the progression of OAN, as well as regarding the optimal amount of soy protein might be drawn in the future.

- Measuring prostanoid production from the series 1 and 3 during the progression of renal disease would give better understanding of the effects of prostanoids in OAN.

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7. APPENDIX (Details of Methods)

7.1 Mean Glomerular Volume (MGV)

Kidneys removed from phosphate-buffered formalin (PBF) were placed in phosphate-buffered saline (PBS), were embedded in paraffin wax, and were sectioned at 5 microns using a Microtome (American Optical 820, Southbridge, Massachusetts). Kidney sections were then placed in xylene to remove the paraffin and stained with hematoxylin and eosin. The kidney sections were stained in Harris's hematoxylin for 5 minutes, washed with deionized water, placed in 1% HCL in deionized water and then placed in 2% ammonia water. Hematoxylin stains nuclei and ribosomes blue. The kidney sections were then washed with deionized water and stained with eosin for 3 minutes, and dehydrated with alcohol. Eosin stains the kidney proteins pink. Cover slips were mounted with cyto seal.

Identification of stained slides of kidney-cross sections was blinded to remove potential bias during analysis by microscopy. Thirty randomly chosen glomeruli per kidney were captured using a camera (Spot Diagnostic Instruments, Inc., Sterling Heights, Michigan) attached to an Olympus BX60 microscope (Olympus Optical Company, Hamburg, Germany). Slides were analyzed using a 20X objective starting from the left edge of the kidney sections and moving to the right. The pictures were sequentially taken using a Spot Advanced software program version 3.0.1 (Diagnostic Instruments Inc, Sterling Heights, Michigan). Captured images of glomeruli were measured using the Image Pro Plus software program (Media Cybernetics, Del Mar, California). A 20X calibration grid was first

opened, and then the file to be analyzed was opened. The largest diameter across each whole glomerulus in the picture was measured. The values for glomerular were exported into a Microsoft Excel file. Each diameter was measured in μm .

MGV was calculated using the method first described by Weibel & Gomez (1962). In order to calculate MGV, an average glomerular diameter was first calculated for each rat and then this number was divided by 2 to give an average radius for each kidney. A mean glomerular area (MGA) was calculated for each rat using the formula as $MGA = \pi r^2$ (the area of a circle). MGV was calculated using the formula as $MGV = 1.25 \times (MGA)^{3/2}$ where the value 1.25 is derived from β/K , where β depends on the shape of the object and is equal to 1.38 for a sphere and K is a distribution coefficient and is equal to 1.10.

7.2 Measurement of Steady-State Protein Levels of Enzymes Involved in Prostanoid Production

7.2.1 Lyophilization of Kidneys

The left kidney from each rat was lyophilized in preparation for western immunoblotting of steady-state protein levels of cPLA₂, COX-1, and COX-2. The left kidney was removed from the -80°C freezer. The frozen kidney was cut up into small pieces and placed into a pre-weighed 15 mL disposable sterile centrifuge tube (Fisher Scientific, cat no 05-539-5, Nepean, Ontario) topped with a lid that had ventilation holes. The tube containing the cut up kidney was weighed again and the initial weight of the frozen kidney was recorded. Tubes

were immersed in liquid nitrogen and then placed in a pre-cooled (-40°C) lyophilizer (Labconco, Model No 4451 F, Kansas City, Missouri) so that samples could begin drying. The freeze dryer was checked periodically and the tubes were removed from the freeze dryer and weighed every few hours or after an overnight drying period until two consecutive equal weights were obtained. The freeze drying process was complete when two consecutive equal tube weights were obtained. If the weight was not equal, the tube was placed back into liquid nitrogen and was returned to the freezer dryer to dry again. Dry kidneys were pulverized using a spatula. The lid without holes was used to replace the lid with holes. Samples were stored at -80°C.

7.2.2 Homogenization of Kidneys

Twenty mg of pulverized kidney was weighed into labeled glass, round-bottomed tubes submerged on ice. This was homogenized in 100 volumes of ice-cold cytosolic homogenization buffer twice for 30 seconds each time using a Polytron homogenizer (Brinkmann Instruments, Type PT 10 20 350D, Rexdale, Ontario) on a speed control setting of 5. Cytosolic homogenization buffer contained 500 mM Tris (Fisher Scientific, Mississauga, Ontario), 0.5 M sucrose, 200 mM ethylene-diamine-tetraacetic acid (EDTA) pH 7.6, 100 mM ethylene glycol-bis (β -aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA) pH 7.6, 0.4 M NaF, 10 mM sodium orthovanadate, 2.5 mg/mL aprotinin, 1 mg/mL leupeptin, 2.5 mg/mL pepstatin, 1 mg/mL soybean trypsin inhibitor (STI), 24 mg/mL 4-(2-aminoethyl) benzene-sulfonyl fluoride (ABSF), β -mercaptoethanol (Fisher

Scientific, Mississauga, Ontario). The cytosolic homogenization buffer was prepared by first mixing all ingredients and then adding 5 M sodium hydroxide (NaOH salt, Fisher Scientific, s318-500, Napean, Ontario) to obtain a final pH of 7.2-7.4 of the solution. Homogenates were transferred into 5mL ultracentrifuge tubes and balanced before a cold rotor was (Beckman Coulter, Inc., model no 50.3TI, Fullerton, California) placed in the pre-cooled (4°C) ultracentrifuge (Beckman, model no L8-80). Samples were spun at 100,000 X g for 35 minutes. The cytosolic fraction remained in the supernatant. A transfer pipette was used to draw off the supernatant and the cytosolic fraction was placed into pre-labeled 2 mL microcentrifuge tubes (Fisher Scientific, cat no 05-408-141, Napean, Ontario) and immediately stored at -80°C.

The pellet was re-suspended in 0.4 mL (20 volumes) of particulate homogenization buffer and the tube was vortexed with a glass rod placed in it to disperse the remaining pellet in the particulate homogenization buffer which consisted of the cytosolic buffer plus 1% Triton X-100. After sitting on ice for 10 minutes, it was vortexed a second time. Tubes were balanced and spun at 100,000 X g for 35 minutes again. The particulate fraction was now suspended in the supernatant and was drawn off and placed into a pre-labeled 2 mL microcentrifuge tube (Fisher Scientific). The samples were immediately stored at -80°C.

7.2.3 Total Protein Determination

Total protein was determined using the Bradford Method (Bradford, 1976) in cytosolic and particulate fractions. Wells in 96-well microplates were labeled as blank, standard or sample. Concentrations of standards were made ranging from 0.0625 mg/mL to 1 mg/mL using a 2 mg/mL bovine serum albumin (BSA) stock solution (Sigma, P0834, Oakville, Ontario). Kidney homogenates were diluted 20X with deionized water. Ten μ L of blank, standard or 20X diluted samples were added to wells in triplicate. Two hundred μ L Bradford Reagent (Sigma, B6916, St. Louis, Missouri) was added to each well using a multi-channel pipette. Then the plate was covered with plastic film and mixed on an orbital shaker (Fisher Scientific, model no 361 Nepean, Ontario) for 15 minutes at room temperature. The plate was read at 595 nm using a microplate reader (Molecular Devices Corporation, SpectraMax 340, Sunnyville, California).

SOFTmax Pro software was used to calculate total protein concentration in the samples based on the equation devised from the standard curve. Final protein concentrations were determined by multiplying by the dilution factor (20-fold diluted samples).

7.2.4 Western Immunoblotting

To separate steady-state protein levels of cPLA₂, COX-1, and COX-2 one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used as has been described (Aukema et al., 2002). This allows for

protein separation as proteins migrate in an electrical field through pores in a gel. The combination of gel pore size, shape, and protein size determines the migration rate of each protein.

Two cleaned glass plates were placed with their sides together and spacers to separate the two plates. The plates were lined up and screwed into place on a gel module (Amersham Biosciences, part of Hoefer miniVE vertical electrophoresis system, 80-6418-77, Buckinghamshire, England) and then deionized water was poured out. A 7.5% separating gel solution consisted of 2670 μL of deionized water, 1250 μL of 1.5M tris aminomethane-hydrochloric acid (tris-HCL), 50 μL of 10% (w/v) SDS (Fisher Scientific, BP166-100), 1000 μL of 40% bis-acrylamide (Fisher Scientific, BP1408-1), 25 μL of 10% (w/v) ammonium persulfate (APS, Fisher Scientific, BP179-25), and 5 μL of N, N, N', N',-tetramethylethylenediamine (TEMED, Fisher Scientific, BP150-20). The latter two ingredients were added just before the solution was added the space in between the plates using a Pasteur pipette until it reached about $\frac{3}{4}$ of the way up the plates. Then the gel was overlaid carefully with deionized water and it was left for 30 minutes to polymerize.

Once the gel was solidified, the water was poured off and a 4% stacking gel that contained 1625 μL of deionized water, 625 μL of 0.5M tris tris-HCL pH 6.8, 25 μL of 10% (w/v) SDS (Fisher Scientific, BP166-100), 250 μL of 40% bis-acrylamide (Fisher Scientific, BP1408-1), 12.5 μL of 10% (w/v) APS (Fisher Scientific, BP179-25), and 2.5 μL of TEMED (Fisher Scientific, BP150-20) was then added on top of the separating gel. Again, the TEMED and APS were added

just before pouring the gel. In order to form lanes to load the protein samples a comb was inserted into the stacking gel and the stacking gel was allowed to polymerize for 45 minutes. The module of the gel was unclipped at the bottom and placed in the electrophoretic chamber filled with running buffer (this running buffer could be re-used up for 10 times) that was made as 10X running buffer and contained 29.0 g of tris base, 144.0 g glycine (Fisher Scientific BP381-1), 10.0 g of SDS, and was made up to 1 L with deionized water. This solution was diluted 10X with deionized water before being used. The new running buffer was poured into the upper compartment of the gel module and the comb was removed and the wells were washed with new running buffer.

cPLA₂ is found in the cytosolic and particulate fractions. COX-1 and COX-2 are only found in the particulate fraction. Cytosolic and particulate fractions were treated with a 2X loading buffer made by mixing 3800 µL of deionized water, 1000 µL of 0.5 M tris (Fisher Scientific, BP-154-1, Napean, Ontario)-HCL (pH 6.8) (Fisher Scientific, A144-500), 800 µL of glycerol (Fisher Scientific BP229-1), 1600 µL of 10% SDS (Fisher Scientific BP166-100), 400 µL of 2-mercaptoethanol (Fisher Scientific, BP176-100), and 400 µL of 1% bromophenol blue (Sigma, B-8026, Oakville, Ontario).

The amount of sample protein loaded was taken from the mid-range of the dose response curve based on our previous study (Warford, 2003). For both cytosolic and particulate cPLA₂ and particulate COX-2, 14 µg of protein was loaded and for particulate COX-1, 16 µg, which is a little higher amount, was loaded due to the low levels of this protein. When the samples were prepared,

they were heated at 100°C for 5 minutes to denature proteins, placed in an Eppendorf 5417C centrifuge at 7000 rpm for 1 minute (Brinkmann Instruments, Mississauga, Ontario), and placed on ice. Then the samples were loaded into each well along with a benchmark pre-stained protein ladder in a separate lane (Invitrogen, cat no 10748-010, Burlington, Ontario). A standard was loaded in duplicate to each gel. The standard was made maxing particulate sample from all rats of each experimental group to control for gel variation across the blot. The protein migrated in running buffer in an electrophoretic chamber (Amersham Biosciences, part of Hoefer miniVE vertical electrophoresis system, 80-6418-77, Buckinghamshire, England) at 200V at room temperature until the dye front just came off the gel. This took approximately 1 hour and 45 minutes.

A hydrophobic polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Hybond-P RPN303F, Buckinghamshire, England) was cut to the proper size and labeled before the dye front ran off the gels. The lower left hand corner of the membrane was cut to indicate the first lane and the membrane was moistened with methanol (Fisher Scientific, A452-4, Napean, Ontario) for 10 seconds and then placed in deionized water on an orbital shaker (Fisher Scientific, model no 361) set at 90 revolutions per minute (rpm) for 7-8 minutes. The water was replaced with transfer buffer and the membrane was put back on the orbital shaker again until the gel was ready to be transferred. The transfer buffer was prepared as 10X transfer buffer that contained 29.0 g tris base (Fisher Scientific, BP154-1), 144.0 g glycine (Fisher Scientific, BP381-1), and was made up to 1 L with deionized water. One hundred mL of 10X transfer buffer was mixed

with 200 mL methanol (Fisher Scientific, A452-4) and 700 mL deionized water before being used.

When the gel was finished running, the gel module was removed from the chamber and the glass plates were separated. The stacking gel was cut off using the spacer and the bottom corner by the first lane also was cut off to indicate the first lane number. The gel was equilibrated in transfer buffer for 5-10 minutes and then was placed in the transfer apparatus (Amersham Biosciences, 80-6418-96, Buckinghamshire, England) against the PVDF membrane in between two pieces of filter paper with the cut corners of the gel and membrane being aligned together. Sponges were added to both sides of the gel so that the gel/membrane/paper was firmly sandwiched in place in the transfer apparatus. The transfer apparatus filled with transfer buffer was placed in a transfer chamber which was filled with deionized water. These proteins were transferred in the 4°C fridge for approximately 2 hours at 375 mA.

After transfer, the membrane was removed from the apparatus and placed in 5% skim milk solution prepared by adding 0.5 g skim milk powder to 10 mL 0.1% tris base solution (TBS/Tween). The membrane was then blocked for one hour in a shallow covered dish on a rocker (Boekel Scientific Rocker II, model 260350, Feastville, Pennsylvania) at room temperature. The solution then was poured off the membrane and was covered with the primary antibody solution.

The primary antibody was prepared in 2% skim milk solution made with 0.2 g skim milk powder in 10 mL TBS/Tween. The concentration of the primary antibody was 1:250 for all proteins, which were cPLA₂ (Santa Cruz Biotechnology,

N-216, antihuman rabbit polyclonal immunoglobulin G (Ig G), cat. no. sc-438, California, USA), COX-1 (Cayman Chemical Company, anti-ovine mouse monoclonal antibody, cat. no. 160110, Ann Arbor, Michigan), and COX-2 (Cayman Chemical Company, anti-mouse rabbit polyclonal antibody, cat. no. 160106). These primary antibody solutions were added to the blocked membrane, the dish was covered and placed on a rocker (Sanyo Gallenkamp, cat. no. IH-370, APP IB 1684, Loughborough, Leicestershire, England) in the 4°C walk-in fridge overnight.

The next morning, the primary antibodies were removed and stored for future use. The membranes then were washed 3 times as follows: 3 X 5 min for both cytosolic and particulate cPLA₂ and 3 X 10 min for both COX-1 and COX-2, therefore, total 15 min and 30 min, respectively in TBS/Tween.

The secondary antibody solutions were prepared in TBS/Tween and added to the membranes, covered and placed on a rocker for one hour at room temperature. The secondary antibody, which was conjugated to horseradish peroxidase, was made using TBS/Tween. Anti-rabbit IgG was used for cytosolic and particulate cPLA₂ and COX-2 at 1:20,000 concentration (Sigma, cat no A-0545). Anti-mouse IgG was used for COX-1 at 1:20,000 concentration (Sigma, cat no A-3682). The membranes were washed again with the same length of time as it was with primary antibody with TBS/Tween after one hour and then they were ready to be developed.

Two chemiluminescent substrates for horseradish peroxidase (Fisher Scientific, ChemiGlow, cat. no. 2900811, Napean, Ontario) were mixed with

deionized water in a ratio of 1:1:4 for 5 minutes. The membrane was placed on plastic wrap and the chemiluminescent mixture was distributed over the membrane and let sit for 5 minutes. After 5 minutes, the membrane covered with saran wrap was developed in using a digital imaging system (Alpha Innotech, San Leandro, California) and analyzed using computer software (AlphaEase FC).

The integrated density value (IDV) was calculated and used as a measure of the intensity of the protein band. In order to obtain IDV, the background pixels were subtracted from the protein band pixels. The IDV value was calculated by dividing the average IDV of the duplicate standards loaded on each gel. IDV are arbitrary units expressing band intensity.

7.3 Measurement of Kidney Function

7.3.1 Urine Creatinine

Urinary creatinine was measured using a colorimetric assay based on the formation of a yellow/orange color when creatinine reacts with alkaline picrate. The creatinine-picrate color fades faster than the color of other interfering substances that form color complexes with picrate when treated with an acidic solution. The amount of creatinine is proportional to the difference in color intensity, which was measured at 500 nm before and after adding an acidic solution. Therefore, the more creatinine in the sample, the stronger the color becomes.

Standards were made ranging from 1 mg/dL to 4 mg/ dL. Urine samples were diluted 20X with deionized water. Standard, sample or blank were mixed

with 1 mL of a picric acid solution made by mixing 2 volumes of 0.05 M sodium phosphate and 0.05 M sodium borate solution with 2 volumes of 4% aqueous SDS (Fisher Scientific, BP166-100) and 1 volume of 1.3% picric acid (Sigma, cat no P6744-1GA) in a 1 mL cuvette. The standards and samples were incubated for 45 minutes at room temperature after the picric acid solution was added to the cuvettes. Then, the absorbance of each cuvette was then read at a wavelength of 500 nm using a spectrophotometer (Milton Roy, Spectronic 3000 Array). Then, 25 μ L of a 60% acetic acid (Fisher Scientific, A38-212) solution was added to each cuvette. The standards and samples were left to incubate for 6 minutes at room temperature and then read again at a wavelength of 500 nm using the spectrophotometer (Milton Roy, Spectronic 3000 Array).

The final absorbance was calculated as follows: Final absorbance = Pre-acid absorbance – Post-acid absorbance. A standard curve was made in Microsoft Excel based on the final absorbance of each concentration of standard. The concentration of creatinine in the urine samples was calculated using the equation from this standard curve.

7.3.2 Serum Creatinine

Serum creatinine was determined using a colorimetric assay. The same principle of the assay was used to measure serum creatinine as was used to measure urine creatinine.

Serum samples were spun in the centrifuge (Eppendorf, Model No 5417C, Hanmburg, Germany) at 11, 000 g for 6 minutes so that any fat in the serum

would come to the top and it was removed. Then the serum was transferred to a clean 0.5 mL microcentrifuge tube (Fisher Scientific, cat no 05-408-128, Nepean, Ontario). Creatinine standard was made from a creatinine standard stock solution (20 mg/dL, Cayman Chemical Company, cat no 10005314, Ann Arbor, Michigan) ranging from 0.05 mg/dL to 10 mg/dL.

Twenty μL of standard, serum sample, or blank were added in triplicate to wells of a 96-well microplate. Two-hundred μL picric solution made by mixing 2 volumes of the 0.05 M sodium phosphate and 0.05 M sodium borate solution with 2 volumes of 4% aqueous SDS (Fisher Scientific, BP166-100) and 1 volume of 1.3% picric acid (Sigma, cat no P6744-1GA) was added to each well using a multi-channel pipette. The plate was read at 500 nm in the microplate reader (Molecular Devices Corporation, SpectraMax 340, Sunnyville, California). A 15% acetic acid solution was made by mixing 1 mL of 60% acetic acid solution (Fisher Scientific, A38-212) with 3 mL of picric acid solution. Twenty μL of this solution was then added to each well using a multi-channel pipettor. The plate was again read at 500 nm in the microplate reader.

The final absorbance was calculated as follows: Final absorbance = Pre-acid absorbance – Post-acid absorbance. Again a standard curve was made in Microsoft Excel based on the final absorbance of each concentration of standard. The concentration of creatinine in the serum samples was calculated using the equation from the standard curve.

7.3.3 Creatinine Clearance

Creatinine clearance was then calculated using the equation: {Concentration of creatinine in urine (mg/dL) X Urine volume (mL)/time (min)}/Concentration of creatinine in serum (mg/dL).

7.4 Prostanoid Production and COX Activity

Sixty mg of lyophilized left kidney from each rat was homogenized in 1.7 mL fresh Tyrodes buffer (Sigma, St. Louis, Missouri). After homogenization, 17 μ L of 1% Triton X-100 was added, vortexed for 10 seconds and the homogenate was placed on ice for 30 minutes. During this 30 minute time period the homogenate was vortexed for 10 seconds every 10 minutes. Twenty μ L of homogenate was stored at -80°C for protein determination using the Bradford assay (Bradford, 1976). One hundred and eighty μ L of the homogenate was aliquoted in duplicate into microcentrifuge tubes containing 20 μ L Tyrodes buffer with either 1% ethanol as vehicle or 0.1 μ M SC560 (Cayman, Ann Arbor, Michigan), which is a COX-1 selective inhibitor and incubated under the following conditions: (1) 0 min with no inhibitor for determination of endogenous levels of prostanoid production; (2) 60 min incubation at 37°C with no inhibitor for determination of steady-state in vitro prostanoid production; (3) 10 min incubation at 37°C with no inhibitor for determination of total COX activity; (4) 10 min incubation at 37°C with 0.1 μ M SC560 (Cayman, Ann Arbor, Michigan) for determination of COX-2 activity. COX-1 activity was determined by the difference between total COX (condition 3) and COX-2 (condition 4) activities.

The incubation conditions were determined from previous time course studies which demonstrated that the production of prostanoids is linear for the first 10 min of incubation, that steady-state levels of prostanoids are achieved by 30-40 min of incubation, and that a concentration of 0.1 μM SC560 inhibits more than 90% of COX-1 activity but does not inhibit COX-2 at all (Warford-Woolgar et al., 2006).

After the incubation time, reactions were stopped by adding 800 μL of ice-cold 5 mmol/L acetylsalicylic acid (ASA) to the sample incubation, vortexing and centrifuging at 12,000 x g at 4°C for 5 minutes. The supernatant was removed and stored at -80°C for determination of PGE₂, 6-keto PGF_{1 α} , (stable metabolite of PGI₂), and TXB₂, (stable metabolite of TXA₂), using commercial enzyme immunoassay kits (Cayman, Ann Arbor, Michigan).

Samples were diluted as follows: 6-keto PGF_{1 α} 250X, 1000X, and 1200X for condition 1, 2 and 3, and 4, respectively; PGE₂ 500X and 1000X for condition 1 and condition 2-4, respectively; and TXB₂ 100X for condition 1-4. Standards ranging from 0.008 to 1 ng/mL, sample, tracer, and antibody were loaded on a plate. When finished plating, the plate was covered with plastic film and incubated on a shaker for 18 hours covered with a box at the following temperature: PGE₂ and 6-keto PGF_{1 α} at 4°C and TXB₂ at room temperature. After the incubation time, the plate was washed with 200 μL of wash buffer 5 times. Then, 200 μL of Ellman's reagent (Cayman, Chemical Company, Ann Arbor, Michigan) was added to each well. Again the plate was incubated on the shaker at room temperature as follows: PGE₂ and TXB₂ for 1 hour and 6-keto PGF_{1 α} for

1.5 hours. The plate was read in the microplate reader (Molecular Devices Corporation, SpectraMax 340, Sunnyville, California) at a wavelength of 412 nm. Prostanoids were expressed relative to protein content of the homogenate.

Appendix 7.5 Effects of dietary soy protein on urine and serum parameters in obese *fa/fa* Zucker rats^{1,2}

	In CTL	In SOY	fa CTL	fa SOY	Main Effects
Urine Volume (mL)	3.82 ± 0.66	5.94 ± 0.99	6.25 ± 1.34	8.65 ± 1.15	Diet (<i>P</i> =0.0147) Genotype (<i>P</i> =0.0180)
Urinary Protein (mg/12 hr)	6.37 ± 1.07	10.06 ± 1.39	15.31 ± 4.94	16.28 ± 3.04	Genotype (<i>P</i> =0.0083)
Urinary Creatinine (mg/12 hr)	4.65 ± 0.22	5.66 ± 0.38	3.55 ± 0.53	3.61 ± 0.27	Diet (<i>P</i> =0.0210) Genotype (<i>P</i> <0.0001)
Serum Creatinine (mg/dL)	0.44 ± 0.03	0.49 ± 0.02	0.39 ± 0.04	0.43 ± 0.02	No effect
Creatinine Clearance /100 g Body Weight (mL/min/100 g)	0.015 ± 0.002	0.016 ± 0.001	0.014 ± 0.002	0.012 ± 0.001	No effect

¹ Data is presented as mean ± SEM (n=10/group, except Serum Creatinine, and Creatinine Clearance/100g Body Weight where n=8 for In CTL group).

² Abbreviations: In CTL, lean rats fed control diet; In SOY, lean rats fed soy protein diet; fa CTL, *fa/fa* rats fed control diet; and fa SOY, *fa/fa* rats fed soy protein diet