TRANS 10, *CIS* 12 CONJUGATED LINOLEIC ACID (*T*10, *C*12 CLA) IS THE ISOMER RESPONSIBLE FOR THE BENEFICIAL EFFECT OF DIETARY CLA IN THE KIDNEYS OF OBESE, INSULIN-RESISTANT RATS

BY

DIELLE JANET HERCHAK

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

We have shown that a mixture of CLA isomers improves oral glucose tolerance and reduces glomerular hypertrophy in the kidneys of obese insulin-resistant *fa/fa* Zucker rats. Therefore, to investigate which CLA isomer is responsible for these effects, 6 week old male *fa/fa* Zucker rats and lean controls were given diets supplemented with either 0.4% (4 g/Kg) of the *c*9, *t*11 CLA isomer (designated as 9,11), 0.4% of the *t*10, *c*12 CLA isomer (designated as 10,12), a combination of the two isomers (0.4% *c*9, *t*11 and 0.4% *t*10, *c*12, designated as TOG) or control (0% CLA, designated as CTL) diet for 8 weeks. The *fa/fa* rats on the 10,12 diet ate significantly less than *fa/fa* rats on all other diets, but diet did not have a significant effect on final body weight. The *fa/fa* rats on the 10,12 diet had a significantly lower kidney weight than *fa/fa* rats on the CTL diet. The *fa/fa* rats also had a lower creatinine clearance compared to lean rats indicating impairment of renal function. Mean glomerular volume (MGV) and protein excretion were elevated by 39% ($P<0.0001$) and 164% ($P<0.0001$), respectively, in *fa/fa* vs. lean rats, reflecting early renal changes associated with obesity-related nephropathy. Compared to the control diet, MGV in the *fa/fa* rats was reduced only in rats given the 10,12 isomer alone (20% lower, $P<0.05$). Similarly, protein excretion was elevated in rats given the 9,11 isomer alone or in combination with the 10,12 isomer, but not in those given the 10,12 isomer alone. Oral glucose tolerance was improved in *fa/fa* rats given the 10,12 isomer alone or in combination with the 9,11 isomer. The *fa/fa* rats had a higher protein expression of cytosolic phospholipase A_2 ($cPLA_2$) along with a higher protein

expression of cyclooxygenase-2 (COX-2) compared to lean rats. COX-2 protein expression was significantly reduced in *fa/fa* rats given the 10,12 isomer compared to *fa/fa* rats on the CTL diet (P=0.0198). Therefore, the beneficial effects of dietary CLA appear to be due to the *t*10, *c*12 CLA isomer, which may mediate its protective effects on early obesity-associated nephropathy by improving glucose tolerance and/or reducing eicosanoid production by COX-2.

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LIST OF ABBREVIATIONS

10,12	diet containing 0.4% of the *t*10, *c*12 CLA isomer
9,11	diet containing 0.4% of the *c*9, *t*11 CLA isomer
AA	arachidonic acid
ACR	albumin to creatinine ratio
ANOVA	analysis of variance
APS	ammonium persulfate
BCA	bicinchoninic acid
BMI	body mass index
$BNaO_24H_2O$	sodium borate
BSA	bovine serum albumin
c	cis
Ca^{2+}	calcium
CD	conjugated diene
CLA	conjugated linoleic acid
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
$cPLA_2$	cytosolic phospholipase A_2
CTL	control diet
Cu^{+}	copper I
Cu^{2+}	copper II
$CuSO_4$	copper II sulfate
CVD	cardiovascular disease
CVs	coefficients of variation
cyto $cPLA_2$	cytosolic fraction of cytosolic phospholipase A_2
cyto $cPLA_2$/part $cPLA_2$	ratio of cytosolic fraction cytosolic phospholipase A_2 to particulate fraction cytosolic phospholipase A_2
DGLA	dihomo-gamma-linolenic acid
DM	diabetes mellitus
DM-1	type 1 diabetes mellitus
DM-2	type 2 diabetes mellitus
DN	diabetic nephropathy
EPA	eicosapentaenoic acid
ESRD	end-stage renal disease
fa/fa	obese Zucker *fa/fa*
FGS	focal glomerularsclerosis
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
GFR	glomerular filtration rate
H_2O_2	hydrogen peroxide
HbA_{1c}	hemoglobin A_{1c}
Hg	mercury
IDV	integrated density volume
IgG	immunoglobulin G

LDL	low density lipoprotein
LPS	lipopolysaccharide
mA	milliamps
MGA	mean glomerular area
MGV	mean glomerular volume
MGVs	mean glomerular volumes
mRNA	messenger ribonucleic acid
$Na_2HPO_47H_2O$	sodium phosphate
NaOH	sodium hydroxide
NCEP	National Cholesterol Education Program
NHANES	National Health and Nutrition Examination Survey
OGTT	oral glucose tolerance test
OLETF	Otsuka Long-Evans Tokushima Fatty
part $cPLA_2$	particulate fraction of cytosolic phospholipase A_2
PBF	phosphate-buffered formalin
PBS	phosphate-buffered saline
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PPAR	peroxisome proliferator-activated receptor
PPARs	peroxisome proliferator-activated receptors
PPARα	peroxisome proliferator-activated receptor-alpha
PPARβ	peroxisome proliferator-activated receptor-beta
PPARγ	peroxisome proliferator-activated receptor-gamma
PVDF	polyvinylidene fluoride
rpm	revolutions per minute
RT-PCR	reverse-transcription polymerase chain reaction
SAD	sagittal abdominal diameter
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
STZ	streptozotocin
t	trans
TBS	tris base solution
TCA	trichloroacetic acid
TEMED	N,N,N',N',-tetramethylethylenediamine
TOG	diet containing 0.4% of the *c*9, *t*11 CLA isomer plus 0.4% of the *t*10, *c*12 CLA isomer
tris-HCL	tris (hydroxymethyl) aminomethane-hydrochloric acid
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2
TZD	troglitazone
USRDS	United States Renal Data System
ZDF	Zucker Diabetic Fatty

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INTRODUCTION

Diabetes is a serious and complicated metabolic disorder that can lead to many problems including diabetic nephropathy (DN), or improper functioning of the kidneys (Health Canada, 2003). Conjugated linoleic acid (CLA) is responsible for the amelioration of several diseases and disorders including glucose tolerance in the Zucker diabetic fatty (ZDF) rat, an animal model of diabetes (Houseknecht et al., 1998). Dietary supplementation with a 1.5% mixture of CLA isomers (containing four major isomers) for 8 weeks ameliorates early renal disease progression in obese male fatty (*fa/fa*) Zucker rats (Warford, 2003). The amelioration of early renal disease was linked to the lower protein levels of cytosolic phospholipase A_2 ($cPLA_2$) and peroxisome proliferator-activated receptor-alpha (PPARα) found in *fa/fa* rats fed the CLA mixture (Warford, 2003). As reviewed by Kramer et al., (2004) synthetic mixtures of CLA contain a different composition of isomers compared to foods that naturally contain CLA. It is therefore of interest to determine which CLA isomer is responsible for reducing renal disease progression in this model. The following sections provide background literature on diabetes and the metabolic syndrome, chronic kidney disease, DN, renal injury in the *fa/fa* Zucker rat, CLA and diabetes in human and rodent models, CLA and DN as well as proposed mechanisms of action of CLA in renal disease.

LITERATURE REVIEW

Part I. Introduction

Diabetes

Diabetes mellitus (DM) has reached epidemic proportions and is one of the most serious diseases affecting Canadians today (Health Canada, 2003). Diabetes is a condition that involves improper production of the hormone insulin by the islet cells of the pancreas (type 1), or the inability of peripheral tissues to respond to the insulin produced by the pancreas (type 2). A third type of diabetes, namely gestational diabetes, occurs in 2-4% of all pregnancies and usually disappears shortly after delivery. As a result of the improper secretion and/or utilization of insulin, the cells of the body are not able to take up glucose. This glucose remains in the circulating blood and causes hyperglycemia, or an elevated blood glucose level that in the long term can lead to severe metabolic complications. The causes of type 1 diabetes (DM-1) are unknown and there is no known means of preventing the disease. Affected individuals depend on a form of externally supplied insulin in order to normalize glucose uptake. On the other hand, physical inactivity and improper nutrition can lead to obesity, and are important factors in the onset as well as the progression of type 2 diabetes (DM-2). This is the most common type of diabetes, as it comprises 90% of all cases. The prevalence of DM-2 in Canada is expected to increase due to the aging population as well as the unhealthy lifestyles adopted by many Canadians (Health Canada, 2003). The good news is that the risk of getting DM-2 can be

greatly diminished by maintaining a healthy lifestyle that includes exercise and proper nutrition.

It is estimated that two and a quarter million Canadians have diabetes, and approximately one third of these adults are undiagnosed (Health Canada, 2003). About 10% of Canadians aged 65 and older have diabetes. This number is closer to 3% for people aged 35-64. Diabetes is the seventh leading cause of death in Canada and 40% of people with diabetes will develop long-term complications associated with the disease. Presently, DM and its complications amount to approximately 9 billion dollars annually in health care costs (Health Canada, 2003).

DM-2 was once thought of as an adult-onset disease, but today that is no longer the case. A review by Rosenbloom et al. (1999) indicates that among youth, DM-2 is now considered "an emerging epidemic", especially for certain ethnic groups such as the Pima Indians and First Nations people. The incidence of DM-2 in African American, Mexican American, Libyan Arab and Japanese children is also on the rise. More young people are now diagnosed with this disease as a result of changing food patterns and increasing obesity rates (Rosenbloom et al., 1999).

The long-term complications of diabetes such as high blood pressure, amputation of the lower limbs, retinopathy, neuropathy, nephropathy, heart disease, and stroke are serious and can lead to premature death (Health Canada, 2003). DN is of particular importance as the risks of cardiovascular disease (CVD), retinopathy and other complications are higher in patients with

renal disease than in those without renal disease (National Kidney Foundation, 2002). Also, patients with chronic kidney disease are considered the "highest risk group" for CVD events (National Kidney Foundation, 2002). Chronic kidney disease is also by far the most costly complication of diabetes if it progresses to end-stage renal disease (ESRD), as the estimated annual cost in Canada of caring for one patient with DM-2 related ESRD is $63,045 (O'Brien et al., 2003). This cost represents a weighted average for people on hemodialysis or peritoneal dialysis, and also includes those who have received a renal transplant (O'Brien et al., 2003).

The link between diabetes and kidney disease has been studied extensively and it is well known that diabetes is the number one cause of ESRD in Canada and the U.S. (United States Renal Data System, USRDS, 2004). In order to study early dietary prevention strategies of kidney disease, it is necessary to evaluate the pre-diabetic state, before the metabolic abnormalities have presented themselves in full. A research area that is gaining new interest is the influence of the metabolic syndrome on kidney function and kidney disease progression.

The Metabolic Syndrome

Before the development of clinical overt DM-2, insulin resistance and subsequent abnormalities in glucose metabolism are present (Goldstein, 2003). These characteristic metabolic abnormalities, along with several other defined characteristics are known collectively as the metabolic syndrome. According to

the Third Report of the National Cholesterol Education Program (NCEP, 2002), the term metabolic syndrome or "syndrome X" is used to describe the co-existence of several metabolic characteristics, namely abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance, and a prothrombotic and proinflammatory state. In order to be clinically diagnosed as having the metabolic syndrome, a person needs to have 3 or more of the criteria listed in **Table 1** (NCEP, 2002). The metabolic syndrome is recognized as an independent risk factor for the development of DM-2, separate from characteristics such as impaired glucose tolerance (Lorenzo et al., 2003). Since DM-2 takes years to develop after signs of the metabolic syndrome are present (Goldstein, 2003), the "pre-diabetic" state of the metabolic syndrome can be viewed as a warning sign and thus, an intervention can be initiated at this stage.

Using data obtained from the Third National Health and Nutrition Examination Survey (NHANES III 1988-1994), Ford et al. (2002) reported that the age-adjusted prevalence of the metabolic syndrome in U.S. adults was 23.7%. Overall, the prevalence increased with increasing age, such as 6.7% in the 20-29 year old age group, and 43.5% among the 60-69 year olds. The criteria used to define the metabolic syndrome in this study were those defined in the Third Report of the NCEP (2002). The authors commented that the present-day prevalence of the metabolic syndrome in the U.S. population is most likely higher than what was estimated from this study due to the usage of older data (Ford et al., 2002).

Table 1: Risk factors for the metabolic syndrome [1]

Metabolic Syndrome Risk Factor	Defining Level for Men	Defining Level for Women
Abdominal Obesity (waist circumference)	>102 cm	>88 cm
Triglycerides	≥1.69 mmol/L	≥1.69 mmol/L
HDL Cholesterol	<1.03 mmol/L	<1.28 mmol/L
Blood Pressure	≥130/85 mmHg	≥130/85 mmHg
Fasting Glucose	≥6.1 mmol/L	≥6.1 mmol/L

[1] As defined by the National Cholesterol Education Program. (2002). Third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III) final report. *Circulation, 106*(25), 3143-3421.

The metabolic syndrome is not only a health concern for the adult and aging population, but is now becoming a problem young people as well. A for study conducted in Quebec, Canada found that the overall prevalence of the metabolic syndrome in youth was 11.5% (Lambert et al., 2004). The criteria used to define the metabolic syndrome in this study was comparable to the criteria published by NCEP in 2002, defining what constitutes the metabolic syndrome in adults (Lambert et al., 2004). This prevalence is of great concern considering the participants in the study were only 9, 13 or 16 years of age.

It is important to consider the metabolic syndrome in relation to kidney disease because just as diabetes can lead to DN (Health Canada, 2003), the metabolic syndrome is associated with an increased risk of chronic kidney disease (Abrass, 2004). In a study of more than 6000 U.S. adults who participated in the NHANES III, it was concluded that people with the metabolic syndrome (as defined according to NCEP in 2002) had increased odds of 2.60 and 1.89 of developing chronic kidney disease and microalbuminuria, respectively, compared to their counterparts who did not have the metabolic syndrome (Chen et al., 2004). The risk of chronic kidney disease and microalbuminuria also increased as the number of components of the metabolic syndrome increased (Chen et al., 2004).

The metabolic syndrome and its association with kidney disease seems to present itself in childhood as well (Csernus et al., 2005). These researchers concluded that obese children (median age of 12.9 years) had a significantly higher urinary albumin to creatinine ratio (ACR) compared to children of normal

weight. Among the obese children, the presence of several cardiovascular risk factors, namely fasting hyperinsulinemia, impaired glucose tolerance, and hypercholesterolemia were all associated with a significantly higher urinary ACR compared to the absence of these risk factors (Csernus et al., 2005).

It is evident from these studies that strategies to delay or prevent chronic kidney disease must be started well before diabetes is clinically diagnosed. Specifically, these strategies need to be initiated in the early stages of obesity and insulin resistance. There is also a need to consider youth and adolescents in these studies, as the obesity epidemic, and more recently obesity-associated kidney damage, is now present in young children (Csernus et al., 2005).

In order to better understand how and when to implement dietary intervention in kidney disease, the definition and stages of chronic kidney disease first need to be discussed in more detail. The following sections will first discuss kidney disease in general terms, and then more specifically in relation to diabetes.

Chronic Kidney Disease

Chronic kidney disease is defined as kidney damage for $\geq$3 months, as evidenced by structural or functional abnormalities, or glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for $\geq$3 months (National Kidney Foundation, 2002). Five stages of chronic kidney disease have been identified (**Table 2**). Stage 5 represents complete kidney failure, which is also known as ESRD. This stage is defined as a GFR <15 mL/min/1.73 m^2. At this point, dialysis or transplantation is

Table 2: The 5 stages of chronic kidney disease [1]

Stage	Description	GFR [2] (mL/min/1.73 m^2)
1	Kidney damage with normal or increased GFR	≥90
2	Kidney damage with mild decreased GFR	60-89
3	Moderate decreased GFR	30-59
4	Severe decreased GFR	15-29
5	Kidney failure	<15 (or dialysis)

[1] Adapted from the National Kidney Foundation. (2002). K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *American Journal of Kidney Disease, 39*(Suppl 1), S1-S256.

[2] GFR = glomerular filtration rate

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). Aside from ESRD, the other major outcome of chronic kidney disease is CVD (National Kidney Foundation, 2002). In fact, patients with chronic kidney disease are more likely to suffer CVD events than kidney failure. CVD is also the leading cause of death in patients with kidney failure. Dialysis patients ages 25-35 have a mortality risk from CVD that is 500 fold higher compared to the general population (National Kidney Foundation, 2002).

It is important to note that most kidney diseases tend to progress and worsen with time (National Kidney Foundation, 2002). The risk of developing kidney failure depends on the level of kidney function at the time chronic kidney disease is discovered, as well as the rate of decline of kidney function (National Kidney Foundation, 2002). If kidney disease is caught early, and the rate of decline of kidney function can be delayed even just slightly by dietary intervention, this could potentially delay the diagnosis of ESRD and subsequent risk of CVD.

Diabetic Nephropathy (DN)

The importance of diabetes in relation to kidney disease cannot be ignored. For each 1 million people living in the U.S. there were 1,435 people diagnosed with ESRD in 2002, and diabetes was the primary diagnosis in 44% of these ESRD cases (USRDS, 2004). The number and proportion of ESRD cases will rise dramatically over the next 10 years as the number of people with

diabetes is expected to double (Loon, 2003). Diabetes is the number one cause of ESRD in industrialized countries (USRDS, 2004), and thus, the importance of delaying and/or preventing kidney disease needs to be emphasized in research studies.

DN is characterized by the presence of proteinuria along with a progressive decline in kidney function (Loon, 2003). Albumin is the specific protein that is a sensitive marker for several types of chronic kidney disease, including DN (National Kidney Foundation, 2002). It is recommended that the standard measurement of albuminuria in patients with diabetes is through an un-timed, spot urine sample, and is expressed as an ACR (National Kidney Foundation, 2002). The terms microalbuminuria (30-299 mg/g creatinine) and macroalbuminuria (>300 mg/g creatinine) are used to define the amount of albumin in the urine (Loon, 2003).

GFR is the best indicator of level of kidney function (National Kidney Foundation, 2002). There is no easy clinical method for measuring GFR (Loon, 2003). Because of this, kidney failure often goes undetected until a patient has lost more than 50% of normal kidney function (Loon, 2003). GFR can be estimated from a prediction equation that calculates estimated creatinine clearance (National Kidney Foundation, 2002). The Cockcroft-Gault formula (**Figure 1**) for estimated creatinine clearance is the most widely used and accounts for age, weight and sex (Loon, 2003). It is important to note that this formula only gives a valid estimation of creatinine clearance when serum creatinine is relatively stable (Loon, 2003).

$$\text{Estimated Creatinine Clearance} = \frac{(140 - \text{age in years}) \times (\text{weight in Kg})^*}{\text{plasma creatinine (mg/dL)} \times 72}$$

*For females, this value is further multiplied by 0.85

Adapted from Loon (2003).

Figure 1. The Cockcroft-Gault formula for estimating creatinine clearance

In a review article by Loon (2003), the pathogenic mechanisms of DN were discussed. The author states that many mechanisms may be involved in damaging the kidney and they appear to be additive in their negative effects. However, the individual susceptibility to such pathogenic mechanisms varies, and is likely a result of genetic predisposition (Loon, 2003). Systemic hypertension occurs in approximately 60-65% of patients with diabetes (Health Canada, 2003) and results in intra-glomerular hypertension, that in turn leads to hyperfiltration causing damage to the kidneys (Loon, 2003). Proteinuria contributes to kidney damage perhaps by the release of inflammatory cytokines. Hypercholesterolemia, cigarette smoking and genetic factors also seem to add to the disease process. Hyperglycemia is associated with progression of DN as well. Studies done in patients with DM-1 or DM-2 indicate that nephropathy occurs more often when the hemoglobin A_{1c} (HbA_{1c}) is >7%. Nutritionally, high protein diets increase the GFR and therefore also may add to the pathogenic mechanisms of DN (Loon, 2003).

The similarities and differences of DN in type 1 and type 2 DM will now be discussed in order to better understand the disease.

DN in Type 1 vs. Type 2 Patients

In contrast to patients with DM-2, the most important structural changes in the kidney that occur in patients with DM-1 who have DN involve the glomerulus (Dalla Vestra & Fioretto, 2003). In these patients, glomerular lesions are present before the onset of clinical manifestations of DN. Glomerulopathy is

characterized by the thickening of the glomerular basement membrane (GBM) and mesangial expansion. Other structural abnormalities that may be present include glomerular enlargement, tubular basement membrane thickening, tubular atrophy, interstitial expansion, afferent and efferent arteriolar hyalinosis, and the thickening of Bowman's capsule. Mesangial fractional volume [Vv(mes/glom)] is used to measure the expansion of glomerular mesangium and represents the proportion of the cross-sectional area of the glomerular tuft made up by mesangium. In DN, the lesions caused by mesangial expansion are what lead to loss of GFR. An expanded mesangium restricts the lumen of the glomerular capillary and reduces filtration surface. The clinical manifestations of DN are related to the expansion of the mesangium (Dalla Vestra & Fioretto, 2003).

In patients with DM-2, renal disease has been described as more complex than in patients with DM-1 (Dalla Vestra & Fioretto, 2003). It has been estimated that approximately 10-30% of DM-2 patients with proteinuria have renal diseases not related to their diabetes. Even when other renal diseases are excluded, renal structure in patients with DM-2 is very heterogeneous. They may have more advanced tubulo-interstitial and vascular lesions compared to glomerular lesions that characterize DM-1 nephropathy patients. Many patients with DM-2 that have microalbuminuria have a normal, or near normal glomerular structure. Because of the heterogeneity seen in the kidneys of patients with DM-2, Dalla Vestra & Fioretto (2003) proposed a classification system that included 3 groups: normal or near normal renal structure, typical diabetic nephropathology and atypical patterns of renal injury (Dalla Vestra & Fioretto, 2003).

A review by Ritz (1999) demonstrated that the risk of developing proteinuria was comparable in patients with DM-1 and DM-2. In patients with DN, the rate of loss of GFR as determined by creatinine clearance also was similar amongst these 2 groups. Finally, the chance of being diagnosed with ESRD after the development of proteinuria also was comparable in both type 1 and type 2 diabetic patients (Ritz, 1999).

Primary prevention strategies for DN include tight blood glucose control along with tight blood pressure control (Marshall, 2004). More specifically, the lowest obtainable HbA_{1c} for the individual should be considered the target with regards to blood glucose control for patients with both DM-1 and DM-2. Strict systemic blood pressure control of <140/80 mm Hg for DM-2 is considered the target, while no valid conclusions exist about primary prevention of blood pressure lowering in DM-1. Once microalbuminuria and proteinuria have set in, several management strategies have been shown to be beneficial in delaying the disease process. These include reducing intraglomerular pressure by inhibiting the renin-angiotensin system, control of systemic blood pressure often with multiple drugs, and reduction of protein intake from the usual high levels to 0.8-1.0 g/Kg body weight/day. It is also extremely important to manage cardiovascular risk by focusing on weight loss, exercise, smoking cessation, glucose control, serum lipids, and blood pressure (Marshall, 2004).

Novel approaches in the management DN have also been studied in various animal models of diabetes (Marshall, 2004), including the use of a specific fatty acid in the progression of early DN in the obese *fa/fa* Zucker rat

(Warford, 2003). Data from Dr. Aukema and Dr. Taylor's lab indicates that early dietary intervention with a CLA mixture in this rat model results in smaller kidney weights and lower mean glomerular volumes (MGVs) compared to those fed a control diet. Lower renal $cPLA_2$ and PPARα protein levels in *fa/fa* rats fed CLA may contribute to the amelioration of these pathological signs of renal disease (Warford, 2003). In order to better understand obesity-related kidney disease, the *fa/fa* Zucker rat model of obesity and insulin resistance will be described in more detail.

Renal Injury in the *fa/fa* Zucker Rat

The *fa/fa* Zucker rat is a representative model of the metabolic syndrome observed in humans. The obesity in this model is caused by an autosomal mutation in the recessive *fa*-gene that encodes for the leptin receptor (Zucker & Zucker, 1961; Chua Jr. et al., 1996). Leptin is a hormone that aids with the homeostatic regulation of energy by increasing energy expenditure and suppressing food intake. These *fa/fa* rats display hyperphagia, which in turn leads to abnormal weight gain and obesity (Bray, 1977). These rats are also hyperinsulinemic (by 3-4 weeks of age) and display peripheral insulin resistance. Pancreatic islets are increased in size as well as number. These *fa/fa* Zucker rats show slightly elevated cholesterol levels and hypertriglyceridemia. They display higher levels of serum lipoproteins with a major elevation in the low-density lipoprotein (LDL) fraction. The size and number of adipocytes are also greater. Renal function is impaired as seen by increased urinary protein and a

decreased capacity of the kidney to concentrate waste products (Bray, 1977). Therefore, the *fa/fa* rats exhibit most of the metabolic abnormalities seen in patients with the metabolic syndrome.

A review by Janssen et al. (1999) discusses various rodent models of type 2 DN, and refers to the *fa/fa* Zucker rat as one of the oldest models for this particular disease. This rat model is said to develop proteinuria and focal segmental glomerulosclerosis (FSGS) that eventually leads to renal failure. Metabolic and/or hemodynamic factors are believed to play a role in the pathogenesis of the nephropathy observed in this model. More specifically, the authors hypothesized that the early renal damage observed in the *fa/fa* Zucker rat happens primarily as a result of the hyperlipidemia present in this model (Janssen et al., 1999).

Kasiske et al. (1985) studied male *fa/fa* and lean Zucker rats at various ages to examine progression of albuminuria and glomerular damage and to determine any functional changes that occurred in the kidneys of this model. Three groups of rats were used with terminations occurring at 14, 28 and 68 weeks, respectively. Urine collections (24 hr) and blood pressure measurements were performed at 12 weeks of age in group 1, at 24 weeks in group 2, and at 60 weeks in group 3. Mean systolic tail cuff blood pressure was significantly higher in *fa/fa* compared to lean rats at 12, 24 and 60 weeks of age. At 14 and 28 weeks of age, the mesangial matrix was significantly greater in *fa/fa* rats compared to lean controls. By 68 weeks of age, mesangial matrix scores were similar in *fa/fa* and lean rats. The mean glomerular area (MGA) was significantly

greater in obese rats compared to lean littermates at 14 and 28 weeks of age. By 68 weeks of age, the MGA of obese rats was not significantly different from those of lean controls. Obese rats showed proteinuria as early as 12 weeks of age compared to lean controls, and this increased markedly by 24 weeks of age. Inulin clearance was measured at 14 and 28 weeks of age and was significantly reduced in 28 week old *fa/fa* rats compared to lean controls. Focal glomerularsclerosis (FGS) was not present in any *fa/fa* rats at 14 weeks of age, but was present in 7 of 8 *fa/fa* rats by 28 weeks of age and in all *fa/fa* rats at 68 weeks of age. FGS was present in 2 of 7 lean rats at 68 weeks of age, but was not present in younger lean rats. This study demonstrates that in the *fa/fa* Zucker rat, albuminuria, mesangial matrix expansion, and increases in MGA precede the development of FGS (Kasiske et al., 1985).

To study events that lead to renal injury, Coimbra et al. (2000) studied 6 male *fa/fa* Zucker rats at 6, 10, 14, 18, 40 and 60 weeks of age as well as 6 male lean Zucker rats as the controls at 6, 14 and 40 weeks of age. Contrary to the study mentioned above by Kasiske et al. (1985), blood pressure in *fa/fa* rats was normal and comparable to that of lean control rats at all time points up to week 40. Plasma creatinine was used to assess renal function and was not significantly different compared to lean controls up to week 40. Proteinuria was significantly increased in *fa/fa* rats at 40 weeks of age compared to lean controls. As determined by light microscopy, mild progressive FSGS was significantly higher in 40 week old *fa/fa* rats compared to age-matched lean control rats. Tubulointerstitial damage was significantly greater in 40 week old *fa/fa* rats

compared to lean control rats of the same age. *De novo* expression of desmin in podocytes was greater in 6 week old *fa/fa* rats compared to age-matched lean controls, and is a protein indicative of podocyte injury. Glomerular hypertrophy was evident in 14 week old *fa/fa* rats compared to age-matched lean control rats, as measured by MGA. The width of the GBM was measured to see if *fa/fa* rats develop changes to the kidney typical of DN. The width of the GBM in 14 week old *fa/fa* rats was significantly greater than in the 14 week old lean controls. Glomerular monocyte/macrophage counts were 1.8 fold higher in 6 week old *fa/fa* rats compared to lean age-matched controls as determined by immunohistology. This study demonstrates that early injury to podocytes and glomerular monocyte/macrophage influx occur as early as 6 weeks of age in *fa/fa* Zucker rats, and is followed by glomerular hypertrophy. Tubulointerstitial damage occurs secondarily in this model (Coimbra et al., 2000).

To test the hypothesis that early prevention of hyperphagia could protect the glomerulus from early injury, Stevenson et al. (2001) used 5 week old female *fa/fa* Zucker rats and pair-fed them to a group of lean Zucker rats for 5 weeks. Pair-feeding the *fa/fa* rats to the lean rats refers to feeding the same amount of feed to *fa/fa* rats as what was eaten by the lean rats during a known period of time. This differs from having a group of pair-weighed rats which is when rats are restricted in the amount of feed they are given in order to keep their body weight as close as possible to the group of rats with the lowest body weight. In order to pair-feed a group of *fa/fa* rats, the authors measured the amount of feed consumed by lean Zucker rats for 2, 3-day periods during each week from ages

4.5 to 10 weeks. The pair-fed *fa/fa* Zucker rats were given the average amount of feed consumed by the lean Zucker rats in the previous 3 days. An ad libitum fed group of *fa/fa* rats was also included in which *fa/fa* rats were given an unrestricted amount of feed and allowed to consume as much as they desired. All groups were fed a 20% soy protein-based diet (w/w), as their previous work with this diet was shown to minimize nephropathy. They found that pair-feeding the *fa/fa* rats significantly reduced hypertriglyceridemia at 10 weeks of age compared to the *fa/fa* rats fed ad libitum. In pair-fed rats, urinary albumin excretion was not different from the lean rats in weeks 6 through 10, while the urinary albumin excretion was above the normal range in 6 week old *fa/fa* rats fed ad libitum and it continued to rise through to week 10. Pair-feeding significantly reduced the expression of desmin in the glomeruli compared to *fa/fa* rats fed ad libitum, as determined by reverse-transcription polymerase chain reaction (RT-PCR). MGVs were not different among the 3 groups at the end of the study. Since reducing overeating also reduces serum triglycerides, the authors speculated that hypertriglyceridemia leads to glomerular injury (Stevenson et al., 2001). This study is important in that it demonstrates how restriction of feed intake in this model of the metabolic syndrome can improve renal pathophysiology at an extremely early stage, even before histological changes in the glomeruli are present. The next section will explore CLA in more detail, as early dietary intervention with this specific fatty acid has been shown to slow the progression of kidney disease in the *fa/fa* Zucker rat and prevent the deterioration of kidney function (Warford, 2003).

Part II. Conjugated Linoleic Acid (CLA)

CLA

CLA is a term describing a group of positional and geometric isomers of conjugated octadecadienoic acid, derivatives of linoleic acid, a polyunsaturated fatty acid denoted as C18:2n-6 (Belury, 2002). CLA occurs naturally as it is formed in the rumen of certain animals by *Butyrovibrio fibrisolvens*, a fermentative bacteria (Evans et al., 2002). It is also present in the breast milk of human females (Precht & Molkentin, 1999). CLA is found in dairy products as well as in ruminant meats (Belury, 2002). Each double bond in CLA can be found in the *cis* or *trans* configuration, giving a large number of isomers as the positioning of the double bonds can change as well as their configurations. In foods, the predominant isomer is *cis* 9, *trans* 11 (*c*9, *t*11). The next most abundant isomer naturally occurring in food is *t*7, *c*9 followed by *c*11, *t*13; *c*8, *t*10 and *t*10, *c*12 (Belury, 2002).

Jahreis et al. (1999) investigated the amount of *c*9, *t*11 CLA in milk samples from various ruminant and non-ruminant milk samples and found that the highest CLA content was in ewe, followed by cow, goat and finally human milk samples. Sow and mare samples contained negligible amounts of CLA. The concentration of CLA in milk from various ruminant animals varied with season. Decreases in CLA concentration occurred in March in all ruminants studied, with an exception of indoor goats (Jahreis et al., 1999).

Due to high costs and/or lack of availability of purified individual isomers, the majority of scientists to date performing experimental studies using CLA have

used a commercially available synthetic mixture of isomers, containing mostly the *c*9, *t*11 isomer and the *t*10, *c*12 isomer (Belury, 2002). In order to understand the role of CLA in human and animal health, it is necessary to study the isomers separately, so that their individual properties can be evaluated. Much of the conflicting data up to this point is likely due at least in part to the fact that mixtures have been used rather than individual isomers. These mixtures may vary in composition and purity, which makes it difficult to compare results from similar experiments. Using specific CLA isomers in experiments will help determine which isomer is related to particular beneficial, or perhaps harmful effects.

Some of the beneficial properties attributable to CLA include its ability to reduce the incidence of induced carcinogenesis, atherosclerosis and adiposity, and to delay the onset of diabetes (Belury, 2002). CLA has been studied in diabetes as it may improve glucose tolerance through a similar mechanism as that of certain pharmaceuticals. More specifically, CLA is a ligand for peroxisome proliferator-activated receptor-gamma (PPARγ), and may exert its anti-diabetic properties by ligand-binding to this peroxisome proliferator-activated receptor (PPAR), similar to the thiazolidinedione drugs (Belury & Heuvel, 1999). An example of a thiazolidinedione is rosiglitazone, an insulin-sensitizing drug that is able to bind to and activate PPARγ. This transcription factor can then regulate the gene expression and differentiation of adipocytes, and in the case of DM-2, this differentiation correlates with improved insulin sensitivity (Belury & Heuvel, 1999). The mechanism underlying the anti-diabetic effects of the

thiazolidinedione drugs is still under debate (Kim & Ahn, 2004). Thiazolidinediones may improve glucose homeostasis not only by increasing systemic insulin sensitization, but perhaps also by the direct activation of PPARγ on genes involved in glucose disposal (Kim & Ahn, 2004). CLA may also improve glucose homeostasis by similar mechanisms. CLA isomers show geometric and positional specificity when it comes to the activation of peroxisome proliferators-activated receptors (PPARs) (Belury & Heuvel, 1999).

Although many of the studies use experimental animal models to study the anti-diabetic effects of CLA, there are some human studies dealing with CLA and diabetes.

CLA and Diabetes in Humans

CLA is known to have both beneficial health effects, as well as unwanted or even harmful outcomes in humans. It may reduce abdominal fat (Risérus et al., 2001), but may also promote insulin resistance, inflammation, and oxidative stress in humans (Risérus et al., 2002a; Risérus et al., 2002b). In a prospective study including 3157 black and white adults ages 18-30, higher dairy consumption was associated with a lower incidence of the metabolic syndrome among overweight participants, defined as body mass index (BMI) $\geq$25 Kg/m^2 (Pereira et al., 2002). The association held true even when controlling for other dietary factors, physical activity and other possible confounding variables. Since the metabolic syndrome is a known risk factor for both DM-2 and CVD, it is possible that some compound in dairy products can be preventative with regards

to these specific diseases. Within the overweight subgroup, the prevalence of the metabolic syndrome decreased by more than 50% from the lowest to the highest quintiles of dairy consumption, and followed a dose-response pattern. After 10 years, the prevalence of the metabolic syndrome was more than 2/3 lower for overweight subjects in the highest quintile compared to those in the lowest quintile of dairy consumption. The observed effect may be attributed to either vitamins and minerals found in dairy products, alterations in dietary patterns associated with dairy consumption, other confounding factors or the "presence in dairy of unrecognized biologically active components" (Pereira et al., 2002). It is not unreasonable to assume that the unknown compound with biological activity could have potentially been the CLA, as this was not ruled out in any fashion.

Risérus et al. (2001) showed that supplementation with CLA reduced abdominal fat in obese men with signs of the metabolic syndrome compared to controls. Subjects (n=24) were supplemented for 4 weeks with either 4.2 g/day of a CLA mixture or an equivalent amount of olive oil to serve as the placebo. The mean decrease in sagittal abdominal diameter (SAD) after 4 weeks was 0.6 cm in the CLA group. There was no change in the SAD in the control group. SAD, also known as "abdominal height", has recently been shown to be a strong non-invasive marker of insulin resistance in obese men and is measured as the distance between the examination table and the belly at the level of the iliac crest of a patient lying in the supine position (Risérus et al., 2004). No improvements were seen in the CLA group with regards to fasting glucose or insulin levels.

One possible explanation for the observed result is that CLA may have inhibited prostaglandin E_2 (PGE_2) production thereby increasing lipolysis of adipose tissue. CLA also may act as a ligand for PPARs, which are important for energy and adipose tissue metabolism. The small sample size of this study and the fact that no change was observed in other related metabolic parameters shows the need for further studies. Abdominal obesity is however a sign of the metabolic syndrome and CLA demonstrated a significant reduction in abdominal fat in these supplemented adult men (Risérus et al., 2001)

Contrary to the positive effects noted above, CLA may be associated with unwanted outcomes in diabetes. Risérus et al. (2002a) supplemented 60 adult men, 35-65 years of age, with 3.4 g/day of a CLA mixture, the *t*10, *c*12 isomer alone or placebo (olive oil) during a 3 month intervention. They concluded that CLA, specifically the *t*10, *c*12 isomer, induced insulin resistance in those men who had signs of the metabolic syndrome (Risérus et al., 2002a). A follow-up report found that the *t*10, *c*12 isomer significantly increased lipid peroxidation (as measured by urinary 8-iso-prostaglandin $F_{2\alpha}$ and 15-ketodihydro-prostaglandin $F_{2\alpha}$) and C-reactive protein compared with placebo (Risérus et al., 2002b). The increase in 8-iso-prostaglandin $F_{2\alpha}$ in the *t*10, *c*12 group was independently related to insulin resistance and hyperglycemia. They concluded that the *t*10, *c*12 isomer of CLA increases oxidative stress and inflammation in men with signs of the metabolic syndrome (Risérus et al., 2002b). These studies demonstrate that CLA, specifically the *t*10, *c*12 isomer, may exhibit pro-diabetic, pro-oxidative and pro-inflammatory properties when consumed by adult males with the

metabolic syndrome. However, as discussed in the following sections, the relationship between CLA and diabetes varies depending on the species.

CLA and Rodent Models of Obesity and Insulin Resistance

a. Mice

Several studies in mice have proposed that CLA can produce unwanted outcomes. DeLany et al. (1999) reported that feeding various doses of a CLA mixture (0-1.0% CLA w/w) for 39 days as part of a high fat diet (45% Kcal from fat) to male AKR/J mice resulted in lower body weights at day 18 in the 0.75% CLA group and at day 21 in the 1.0% CLA group compared to controls. CLA also reduced body fat content in various adipose depots in a dose-dependent manner without affecting overall energy intake (except for in the 0.25% CLA group which had a higher energy intake compared to other groups). Plasma glucose concentrations were not altered by CLA treatment. Although these results seem beneficial, it is important to note that CLA also increased fasting plasma insulin levels in a dose-dependent manner, and it was significantly higher compared to controls at a dose of 1% CLA. Liver weights also were significantly higher in the group fed 1% CLA compared to controls, as a result of increased lipid accumulation. High insulin levels were not expected with feeding CLA, as a reduction in body weight and in adipose mass is usually associated with improved insulin sensitivity. One possible explanation according to the authors is that if CLA is inducing a lypolytic state, fatty acids will be more available to skeletal muscle and this could lead to insulin resistance (DeLany et al., 1999).

Although decreased body weight should prove to be beneficial for diabetes in that it helps to improve insulin sensitivity, insulin resistance and fatty liver were in fact observed in this study.

A study by Tsuboyama-Kasaoka et al. (2000) revealed that supplementing female C57BL/6J mice with a 1% CLA mixture (w/w) in a 10% fat diet (by Kcal) for up to 8 months, reduced adipose tissue mass (by increasing apoptosis as well as by decreasing cell size), but did not significantly alter energy intake or body weight. Blood glucose levels did not differ between groups. Insulin resistance and hepatomegaly due to steatosis were again present in the CLA group. The increase in plasma insulin concentration was accompanied by a decrease in plasma leptin concentrations in the CLA group. Plasma insulin concentrations became close to normal after leptin treatment, suggesting that leptin deficiency may contribute to insulin resistance. Leptin treatment also ameliorated lipid accumulation in the livers of the CLA group (Tsuboyama-Kasaoka et al., 2000).

Clement et al. (2002) conducted a similar experiment to the 2 studies mentioned above, but fed the CLA isomers separately. Growing female C57BL/6J mice were fed a control diet (sunflower oil), a linoleic acid diet, a *c*9, *t*11 isomer diet or a *t*10, *c*12 isomer diet for 4 weeks. Experimental diets contained 0.4% CLA (w/w) and 2.4% total oil (Clement et al., 2002). These were considered low fat diets as the recommended fat content for a growing rodent is 7% w/w (Reeves et al., 1993). Supplementation with CLA induced a decrease in energy intake, but animals fed the linoleic acid diet also experienced a similar decrease (Clement et al., 2002). Body mass was not significantly different

among the groups. The group fed the *t*10, *c*12 isomer had a decrease in mass of the peri-uteral white adipose tissue. Unfortunately, the *t*10, *c*12 diet was also the only treatment that resulted in liver enlargement (a 3.1 fold increase) and unfavorably modified expression of hepatic genes involved in fatty acid transport and uptake into adipocytes. The *t*10, *c*12 isomer decreased plasma leptin levels. Plasma glucose was not affected by the treatments. The *t*10, *c*12 isomer increased non-fasting plasma insulin concentrations 10 fold compared to mice fed the other 3 diets (Clement et al., 2002). The results from this experiment are similar to the results of DeLany et al. (1999) and Tsuboyama-Kasaoka et al. (2000). A decrease in energy intake as a result of feeding CLA was observed only in the study by Clement et al. (2002). Clement et al. (2002) fed the isomers separately, thus, they were able to conclude that the *t*10, *c*12 isomer appears to be responsible for increasing insulin levels and inducing fatty liver in mice, and that the *c*9, *t*11 isomer does not seem to produce these undesirable effects.

To summarize the effects of CLA in mice, these studies all show consistent findings, in that feeding CLA to mice results in a reduction of adipose tissue mass, but it also increases liver lipid accumulation and insulin resistance. The CLA isomer responsible for these observed effects seems to be *t*10, *c*12 isomer.

b. Rats

Various studies have examined the effects of CLA on glucose and insulin concentrations in ZDF rats (Houseknecht et al., 1998 and Ryder et al., 2001).

These rats were established at the Indiana University School of Medicine (Clark et al., 1983). Breeding pairs donated to the Diabetes Research and Training Center at Indiana University Medical School by Dr. Walter Shaw at the Eli Lilly Company were used to establish an outbred colony. Several of these rats were found to have high blood glucose concentrations. These rats underwent a modified breeding program with some inbreeding and the diabetic trait was genetically transmitted. Most of the ZDF rats were males (Clark et al., 1983). Both ZDF and *fa/fa* Zucker rats have hypertriglyceridemia, elevated cholesterol concentrations, hyperinsulinemia, insulin resistance and nephropathy, but ZDF rats are also hyperglycemic and a model for DM-2.

To study the effects of CLA on glucose tolerance, 6 week old male ZDF and lean rats were fed diets containing no CLA (control), a 1.5% CLA mixture or 0.2% troglitazone (TZD) for 14 days (Houseknecht et al., 1998). CLA and TZD were compared as CLA shares functional similarities and may act in a similar fashion as thiazolidinediones as ligands of PPARγ. At the end of the study, ZDF rats fed CLA had body weights similar to ZDF control rats, but weighed significantly less than ZDF rats fed TZD. ZDF rats on the control diet were hyperglycemic at day 14, compared to lean rats or ZDF rats fed CLA or TZD. CLA as well as TZD attenuated plasma insulin and glucose concentrations in ZDF rats compared to ZDF rats fed the control diet. CLA and TZD attenuated the rise in plasma glucose levels following an intra-peritoneal glucose injection in the ZDF rats. ZDF groups fed either CLA or TZD had lower circulating concentrations of free fatty acids compared to both lean rats and ZDF controls.

To further this research and look at a possible mechanism of action of CLA, the authors assessed PPARγ activation in African green monkey kidney CV-1 cells *in vitro*, a cell line that is commonly used in transactivation studies. Increasing concentrations of CLA induced PPARγ activation in these cells *in vitro*. This demonstrates that CLA may be acting via this specific PPAR. The authors concluded CLA has the potential to act as an insulin-sensitizer through the activation of PPARγ, and may work by mechanisms similar to those of TZD (Houseknecht et al., 1998).

In order to test the isomer-specific properties of CLA, Ryder et al. (2001) fed 6 week old male ZDF rats as well as lean littermates, one of 3 high fat (40% w/w) diets for 14 days. The control diet contained 0.2% CLA and the CLA diets contained a total of 1.5% CLA. The *c*9, *t*11 isomer diet (>90% *c*9, *t*11) contained CLA-enriched butter. The 50:50 isomer diet (*c*9, *t*11 and *t*10, *c*12) contained a synthetic CLA oil mixture, made up predominantly of these 2 isomers. A pair-fed group was also included in which ZDF rats were fed the control diet in an amount analogous to the intakes of the animals on the 50:50 diet, to control for a possible reduction of feed intake by rats on the 50:50 diet as previously observed by this research group (Houseknecht et al., 1998). Feed intake was increased in the *c*9, *t*11 group compared to control, and was lower in the 50:50 group compared to the control and the *c*9, *t*11 groups. Final body weights were significantly higher in the *c*9, *t*11 and control groups compared to the 50:50 and pair-fed groups. ZDF controls and the *c*9, *t*11 group were hyperglycemic compared with lean controls by the end of the study, but the 50:50 group and pair-fed rats maintained

normal glycemia. ZDF control rats were glucose intolerant compared with lean controls as determined by a glucose tolerance test. The *c*9, *t*11 group showed no improvements in glucose tolerance compared with ZDF controls, but the 50:50 group demonstrated improved glucose tolerance. Only an intermediate effect on glucose tolerance was observed in pair-fed animals, so the effects of the 50:50 CLA diet cannot be completely explained by reduced feed consumption. Plasma insulin was lowered equally in the 50:50 and pair-fed groups, but not in the *c*9, *t*11 group compared with ZDF controls (Ryder et al., 2001).

It is evident from the studies in the ZDF rat (Houseknecht et al., 1998; Ryder et al., 2001) that CLA improves glucose tolerance in this rat model of diabetes. A CLA mixture is more efficient than the *c*9, *t*11 isomer alone with regards to anti-diabetic properties (Ryder et al., 2001). Further studies need to be carried out looking specifically at the *t*10, *c*12 isomer to see whether it has anti-diabetic properties when given alone.

Part III. Conjugated Linoleic Acid (CLA) and Diabetic Nephropathy (DN): Proposed Mechanisms

CLA and DN

Feeding a 1.5% (w/w) CLA mixture for 8 weeks to 6 week old obese *fa/fa* Zucker rats results in amelioration of early pathological signs of renal disease (Warford, 2003). Obese *fa/fa* Zucker rats fed CLA had an 11% smaller kidney weight compared to *fa/fa* Zucker rats fed a control diet. Obese *fa/fa* Zucker rats

fed CLA also had a 28% smaller mean glomerular volume (MGV) compared to *fa/fa* Zucker rats fed the control diet. It was hypothesized that modification of enzymes involved in eicosanoid production or modification of PPARs by CLA was associated with ameliorations in renal disease in this model. When these protein levels were measured, it was reported that cyclooxygenase-2 (COX-2), $cPLA_2$, and peroxisome proliferator-activated receptor-β (PPARβ) were elevated in *fa/fa* rats compared to lean rats, while PPARα was reduced. Obese *fa/fa* Zucker rats had 187% higher COX-2 protein levels, 35% higher particulate $cPLA_2$ protein levels and 31% higher PPARβ levels compared to lean Zucker rats. They also had 22% lower PPARα protein levels compared to lean Zucker rats. Feeding CLA significantly lowered both $cPLA_2$ and PPARα in both rat genotypes. Zucker rats fed CLA had 26% lower particulate $cPLA_2$ protein levels compared to Zucker rats consuming the control diet. Feeding CLA to Zucker rats resulted in 21% lower PPARα protein levels compared to feeding the control diet. It was concluded that the amelioration of early renal disease progression in these obese *fa/fa* Zucker rats may have been due to alterations in protein expression of particulate $cPLA_2$. It was suggested that lower particulate $cPLA_2$ expression could be associated with less release of arachidonic acid (AA) from membrane phospholipids, and perhaps less production of thromboxane A_2 (TXA_2), a potent vasoconstrictor. The CLA used in this study was a mixture and was 86.3% pure. It was composed of four major isomers; namely *t*10, *c*12 (30% of total CLA), *c*9, *t*11 (29% of total CLA), *c*11, *t*13 (18% of total CLA) and *t*8, *c*10 (16% of total CLA) (Warford, 2003); thus, the specific isomer responsible for the amelioration

of renal disease still needs to be identified. To understand how specific CLA isomers might ameliorate early renal disease progression, insulin-sensitizing pharmaceuticals and their association with ameliorating DN will be reviewed.

DN and Insulin-Sensitizing Pharmaceuticals

McCarthy et al. (2000) demonstrated that troglitazone, administered to 6 week old ZDF rats (6 mg/g chow for the first 12 weeks and 12 mg/g chow between weeks 13-25) was not only able to attenuate the hyperglycemia and hypertriglyceridemia seen in ZDF control rats, but that this thiazolidinedione also was able to attenuate glomerular volume in these diabetic rats. The degree of glomerular hypertrophy was less in ZDF rats given troglitazone compared with ZDF control rats at all time points (1, 3 and 6 months). Since thiazolidinediones are capable of normalizing hyperglycemia and controlling abnormal lipid metabolism in DM-2, it was hypothesized by the authors that both of these factors could have been responsible for ameliorating glomerular hypertrophy. The authors also suggested that troglitazone may have had a direct effect on the glomerulus (McCarthy et al., 2000).

Imano et al. (1998) proposed that troglitazone could improve microalbuminuria in patients with DM-2 as a result of its effect on vascular tissue. Patients (n=30) with DM-2 and microalbuminuria (urinary ACR ranging from 30-300 mg/g creatinine) participated in the study. They were randomly assigned to one of two groups: treated with 500 mg/day of metformin (n=13) or 400 mg/day of troglitazone (n=17) for 12 weeks. The results indicated that despite similar

reductions in fasting and post-meal glucose levels for both groups, only the troglitazone group showed a reduction in ACR. The average value for ACR at the beginning of the study was 70 mg/g creatinine and was reduced to 40 mg/g creatinine after 4 weeks with troglitazone supplementation. These reduced levels were maintained throughout the 12 week study period (Imano et al., 1998).

Fujiwara et al. (2000) demonstrated that troglitazone lowered urinary protein excretion in female Wistar fatty rats from 24 $\pm$ 7 mg/day to 7 $\pm$ 1 mg/day after 24 weeks of treatment, compared to Wistar fatty rats fed a control diet. These rats are obese and insulin-resistant but do not have overt diabetes. The fatty rats also displayed an elevated serum insulin and serum insulin/glucose ratio compared to lean rats, which were both ameliorated with troglitazone supplementation (Fujiwara et al., 2000).

It is thought that the thiazolidinedione drugs may reduce albuminuria in rat models of diabetes by inhibiting the loss of anionic sites of the GBM (Yamashita et al., 2002). These anionic sites act as a charge-selective filtration barrier and regulate the movement of macromolecules across the GBM. When they are lost, membrane permeability to proteins such as albumin is increased. When streptozotocin (STZ)-treated spontaneous hypertensive rats were treated with either 0.1% troglitazone or 0.001% pioglitazone for 12 weeks, the albumin excretion rate was suppressed compared to STZ-treated spontaneous hypertensive rats fed the control diet. The anionic sites of the GBM in rats treated with either of the thiazolidinedione drugs were arranged regularly and were plentiful, and looked like the anionic sites of the non-diabetic spontaneously

hypertensive control rats. Quantitatively, the number of anionic sites was decreased in STZ-treated spontaneous hypertensive rats given the control diet compared to STZ-treated spontaneous hypertensive rats given either troglitazone or pioglitazone (Yamashita et al., 2002). This study is useful in that it provides a potential mechanism of action of how the thiazolidinedione drugs ameliorate DN.

The thiazolidinedione drugs have been shown to ameliorate signs of DN in various rat models (McCarthy et al., 2000; Fujiwara et al., 2000; Yamashita et al., 2002) and also in humans (Imano et al., 1998). Since CLA shares similarities with the thiazolidinediones (Belury & Heuvel, 1999), it is reasonable to hypothesize that specific CLA isomers also may ameliorate early signs of DN in the *fa/fa* Zucker rat model of obesity and insulin resistance. It is important to find out if it is a single CLA isomer, or a combination of isomers that proves more beneficial as this will allow for greater insight into isomer-specific effects of CLA in dietary treatment of renal disease.

Another area that is gaining interest with regards to kidney disease is the downstream production of eicosanoids from AA. This area will now be explored in more detail first by looking generally at eicosanoid production, and secondly by discussing eicosanoid involvement with renal disease in rat models of obesity and insulin resistance.

Production of Eicosanoids from Cyclooxygenase (COX) Enzymes

Linoleic (18:2n-6) and linolenic (18:3n-3) acid are considered essential as mammals lack the delta 12 and delta 15 desaturase enzymes needed to add a

double bond in the n-6 and n-3 positions, respectively, in the carbon chain of fatty acids (Dupont, 1990). COX is one of the enzymes that produces a range of oxidized products from 20 carbon n-6 and n-3 fatty acids known as eicosanoids. The other enzyme responsible for eicosanoid production from n-6 and n-3 fatty acids is lipoxygenase. Of the 20 carbon fatty acids, there is a higher amount of AA (20:4n-6) in membrane phospholipids compared to either dihomo-gamma-linolenic acid (DGLA) (20:3n-6) or eicosapentaenoic acid (EPA) (20:5n-3). COX also has a higher specificity for AA than for the latter two fatty acids. Under normal circumstances, eicosanoids 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. Eicosanoids derived from EPA tend to oppose or have weaker effects than those derived from AA (Dupont, 1990). The eicosanoids coming from DGLA tend to have anti-inflammatory as well as anti-proliferative properties and therefore milder biological effects compared to AA derived eicosanoids (Fan & Chapkin, 1998).

Eicosanoid Enzymes and Eicosanoid Production in Renal Disease

Dey et al. (2004) measured cyclooxygenase-1 (COX-1) and COX-2 protein expression in renal microvessels of male lean and obese ZDF rats that were 20-21 weeks of age. They found that the expression of COX-1 did not differ between lean and obese ZDF rats, but that COX-2 expression was increased 1.6 fold in obese ZDF, compared to lean Zucker rats. Urinary excretion of thromboxane B_2 (TXB_2) and 6-keto prostaglandin $F_{1\alpha}$ were significantly higher in

obese rats. In contrast, PGE_2 was lower in obese rats compared to lean rats. Some obese rats were given 10 mg/Kg/day of rofecoxib (a COX-2 inhibitor) for 3 weeks in their drinking water. Treatment with this drug did not alter urinary excretion of TXB_2 or PGE_2, but did, however, reduce urinary excretion of 6-keto prostaglandin $F_{1\alpha}$ and ameliorated the glomerulosclerosis observed in ZDF rats (Dey et al., 2004). This study implies that COX-2 is involved in renal disease progression in ZDF rats as inhibiting this enzyme ameliorates histological signs of glomerular injury in this model.

Recently, Komers et al. (2005) 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 and this higher expression of COX-2 became more prominent at 12 weeks of age. In contrast, COX-1 protein expression did not differ between ZDF rats and lean rats at 4 weeks of age. However, at 12 weeks of age, COX-1 protein expression was lower in ZDF rats compared to lean controls. Urinary excretion of PGE_2 and TXB_2 was significantly elevated in ZDF rats versus lean rats at 12 weeks. ZDF rats at 4 and 12 weeks of age had higher kidney weights compared age-matched lean Zucker rats. In 12 week old rats, creatinine clearance was not different between ZDF and lean Zucker rats, but urinary protein was significantly higher in ZDF compared to lean Zucker rats. Wortmannin (100 µg/Kg body weight) was then given by intraperitoneal injection to additional groups of 12 week old ZDF and lean control Zucker rats to examine the role of hyperinsulinemia on renal COX-2, as this drug is an inhibitor of phosphatidyl inositol-3-kinase, an insulin-signaling intermediate. They found that

ZDF rats treated with wortmannin had a lower TXB_2 excretion compared to vehicle-treated ZDF rats. The authors concluded that elevated COX-2 activity appears to be at least partially dependent on insulin (Komers et al., 2005).

A recent article by Xu et al. (2005) concluded 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. In contrast, COX-1 mRNA expression was not altered in any group (Xu et al., 2005). In the male *fa/fa* Zucker rat, the regulation of COX-2 seems to be affected by the angiotensin II-angiotensin II type 1 receptor pathway.

Data obtained from our lab using a mouse model of polycystic kidney disease indicates that protein expression of $cPLA_2$ in both the cytosolic and particulate fractions are higher in diseased male and female mouse kidneys compared to controls at 180 days of age (Aukema et al., 2002). Female mice show increased levels of this protein in the cytosolic and particulate fractions as early as 60 days of age. Protein expression of $cPLA_2$ in both fractions is also significantly increased in the kidneys of male rats with polycystic kidney disease at 70 days of age. Alterations in the COX enzymes in these rodent models of polycystic kidney disease are also evident. 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. Contrary to observations in other rat

models of kidney disease (Warford, 2003; Dey et al., 2004; Xu et al., 2005; Komers et al., 2005), COX-2 protein levels were lower in diseased male rat kidneys compared to controls at 70 days of age (Aukema et al., 2002).

Okumura et al. (2000) measured the urinary excretion of eicosanoids in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a model of DM-2, and compared this to lean control rats. Renal excretion of TXA_2 and Prostaglandin I_2 (PGI_2) increased before marked proteinuria in the OLETF rat. As nephropathy progressed, they maintained a high level of TXA_2 excretion, but the excretion of PGI_2 declined. The altered renal production of these eicosanoids is thought to be associated with progression of DN in this model of DM-2 (Okumura et al., 2000).

It is evident from the above-mentioned studies that enzymes involved in eicosanoid metabolism are altered in kidney disease. They appear to be altered in different manners depending on the rodent model, the age of the animal, and the specific area of the kidney that is being studied (Aukema et al., 2002; Warford, 2003; Dey et al., 2004; Xu et al., 2005; Komers et al., 2005). As well, urinary excretion of eicosanoids is not altered in the same manner in all rats, and varies depending on the model of kidney disease and with progression of the disease (Dey et al., 2004; Komers et al., 2005; Okumura et al., 2000).

CLA can affect enzymes involved in eicosanoid production (Warford, 2003) and thereby may improve kidney disease through this mechanism since eicosanoid production and/or enzymes involved in eicosanoid production are altered in kidney disease (Okumura et al., 2000; Aukema et al., 2002; Dey et al., 2004; Komers et al., 2005; Xu et al., 2005). The potential mechanisms as to how

CLA may affect enzymes involved in eicosanoid production will first be discussed. This will be followed by a discussion of a study where CLA proved to be beneficial in altering eicosanoid production in a rat model of kidney disease.

CLA and Eicosanoid Enzymes/Eicosanoid Production

Belury (2002) suggested that CLA is able to modulate eicosanoid production from AA. The mechanism of how CLA reduces these downstream eicosanoid products can be explained by at least 3 theories. One theory is that CLA can displace AA in phospholipids. This comes from the idea that CLA, like linoleic acid, is metabolized through elongation and desaturation *in vivo*. Elongated and desaturated CLA metabolites have been found in various tissues of both rats and humans. This would result in a diminished AA pool in phospholipids from linoleic acid, and perhaps displacement of AA with conjugated AA. Secondly, CLA may inhibit COX-1, and/or the inducible form, COX-2, at the level of mRNA, protein or activity. Thirdly, CLA or its elongated and/or desaturated products may act as substrates or antagonists for COX, which would lead to a reduction in available enzyme for AA (Belury, 2002).

A review by Banni (2002) indicates that CLA has been shown to undergo delta 6 desaturation, elongation, and delta 5 desaturation, in a similar fashion to linoleic acid while still maintaining the conjugated diene (CD) structure. This leads to a CD structure of 20:4, which has been shown to displace AA in phospholipids (Banni, 2002). This CD 20:4 fatty acid may produce different

eicosanoids through the COX enzyme pathways which could potentially be beneficial in the progression of kidney disease.

In order to explore a mechanism of mammary cancer risk reduction in the rat, Banni et al. (1999) conducted a study in which female Sprague-Dawley rats were fed either a control diet, or a diet containing graded amounts of a CLA mixture (0.5-2% CLA) for 4 weeks. CLA accumulated in mammary fat pads, liver and plasma in a dose-dependent manner with increasing amounts of dietary CLA. Metabolites of CLA, namely CD 18:3 and CD 20:3 (the first desaturation and elongation products of CLA, respectively) also showed a graded increase in mammary tissue and liver from 0.5-2% dietary CLA. Liver and mammary tissue concentrations of linoleic acid were not altered in rats fed CLA, regardless of the level of intake. The first 2 desaturation and elongation products (18:3 and 20:3, respectively) of linoleic acid were significantly lower in mammary tissue of rats fed CLA at a level of 0.5%. Beyond this level, no further reduction in metabolites was seen. AA (20:4) was significantly reduced when rats were fed a level of 0.5% CLA, and reduced even further at a level of 1% CLA. Beyond this, no further reduction was noted. These above-mentioned changes were not seen in the liver suggesting tissue specificity of CLA metabolism (Banni et al., 1999). This research shows that CLA can undergo elongation and desaturation, in a similar manner as linoleic acid, and therefore displace AA, at least in mammary tissue of this rat model. This study examined the mechanism through the quantification of metabolites which are formed before the conversion to AA takes

place. Unfortunately, neither COX enzymes nor eicosanoids were measured in this study.

Various studies have demonstrated that CLA can modulate eicosanoid enzymes and/or eicosanoid production in a variety of tissues and in a variety of species. Nakanishi et al. (2003) fed a 3% (w/w) mixture of CLA isomers (containing approximately 31% *c*9, *t*11 and 32% *t*10, *c*12) or control diet containing soybean oil with no CLA to 8 week old male and female mice 1 week before mating. The same diet (CLA or control) that was fed to the dams was then fed to weaned offspring. They found that PGE_2 in the cerebrums of male offspring fed CLA was significantly reduced at both 25 days and 8 weeks of age. Interestingly, CLA increased COX-1 mRNA by 177% (significant) and COX-2 mRNA by 206% (nearly significant, P=0.06) in 25 day old offspring, but they were not determined at 8 weeks of age (Nakanishi et al., 2003).

A CLA mixture (30 μM of *c*9, *t*11 and *t*10, *c*12) was found to reduce PGE_2 production in lipopolysaccharide (LPS) stimulated murine macrophages, compared to cells treated with AA (30 μM) or cells with no added lipid to serve as control (Iwakiri et al., 2002). CLA also significantly reduced COX-2 mRNA levels in LPS stimulated macrophages compared to cells treated with AA or control cells (Iwakiri et al., 2002). CLA seems to alter PGE_2 production through modulation of COX-2 at the level of mRNA, at least in macrophages.

As evidenced by Urquhart et al. (2002), different CLA isomers, as well as different concentrations of these isomers, affect stimulated production of eicosanoids differently. When human saphenous vein endothelial cells were

incubated with individual CLA isomers (100 μM) and stimulated with a calcium ionophore, the *c*9, *t*11 isomer inhibited production of eicosanoids [namely PGI_2, TXB_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), PGE_2 and prostaglandin D_2 (PGD_2)] by 50% while the *t*10, *c*12 isomer stimulated production of these eicosanoids by 200%. A 50:50 mixture of the 2 isomers inhibited the calcium ionophore stimulated eicosanoid production of the eicosanoids mentioned above in a dose-dependent fashion (50-200 μM). Interestingly, both of these individual isomers had inhibitory effects on calcium ionophore stimulated eicosanoid production when tested at a level of 50 μM. Both the CLA mixture (100 μM) and individual isomers (50 μM) inhibited basal production of $PGF_{2\alpha}$, a major eicosanoid produced by this cell line. The CLA mixture (100 μM) significantly increased incorporation of AA into diacylglycerol phosphatidylinositol and the *c*9, *t*11 isomer (50 μM) significantly increased incorporation of AA into diacylphosphatidylcholine. Neither the CLA mixture (100 μM) nor the separate isomers (50 μM) were found to alter protein expression of COX-1 in these cells under basal conditions or stimulation (Urquhart et al., 2002). This study demonstrates the complexity of the interrelationships between dose, isomer, and stimulated versus basal production of eicosanoids and alterations in this eicosanoid production by CLA.

CLA was found to ameliorate the progression of renal disease in the Han:SPRD-*cy* rat model of polycystic kidney disease, when compared with a control diet of corn oil (Ogborn et al., 2003). A CLA mixture was fed for 8 weeks, at a level 0.67% (w/w) of diet, and contained mostly the *c*9, *t*11 isomer as well as other isomers, but a relatively small amount of the *t*10, *c*12 isomer. CLA

ameliorated renal fibrosis and reduced macrophage infiltration in the diseased rats. CLA also reduced the *ex vivo* release of PGE_2 from the kidneys of diseased rats, as well as from healthy controls (Ogborn et al., 2003). CLA seems to be beneficial in ameliorating kidney disease in this model, possibly by reducing the renal release of the specific eicosanoid, PGE_2.

In summary, CLA appears to decrease PGE_2 in a variety of tissues and cells from both animal and human origin as mentioned above. PGE_2 is not the only eicosanoid modified by CLA, as this fatty acid is capable of altering stimulated *in vitro* production of other eicosanoids as well (Urquhart et al., 2002). Effects on CLA on COX-1 and COX-2 enzymes are variable and include increases, reductions, or no change in COX levels at the level of mRNA or protein (Warford, 2003; Nakanishi et al., 2003; Iwakiri et al., 2002; Urquhart et al., 2002).

RATIONALE

Altered renal eicosanoid production is observed in kidney disease (Dey et al., 2004; Komers et al., 2005; Okumura et al., 2000). Enzymes involved in eicosanoid production are also altered in models of renal disease (Warford, 2003; Dey et al., 2004; Komers et al., 2005; Xu et al., 2005; Aukema et al., 2002). CLA is able to alter eicosanoid production in a variety of tissues and cells (Belury, 2002; Nakanishi et al., 2003; Iwakiri et al., 2002; Urquhart et al., 2002), perhaps through modulation of COX enzymes (Belury, 2002; Nakanishi et al., 2003; Iwakiri et al., 2002). More importantly, CLA has been shown to decrease renal production of PGE_2 and ameliorate disease progression in a rat model of polycystic kidney disease (Ogborn et al., 2003). Reducing the production of downstream eicosanoids from AA with CLA may prove beneficial in ameliorating other types of kidney disease, such as kidney disease associated with the metabolic syndrome. The *fa/fa* Zucker rat is an appropriate model to study renal disease related to the metabolic syndrome (Toblli et al., 2004).

CLA is also able to improve oral glucose tolerance in the ZDF rat (Houseknecht et al., 1998). It is of interest to determine whether CLA also can improve oral glucose tolerance in the *fa/fa* Zucker rat model, and if so, which specific isomer/isomers/isomer mixture shows the most promising results. Improved metabolic control with CLA may prove beneficial in ameliorating early renal disease progression.

The *fa/fa* Zucker rat model displays elevated COX-2 levels (Warford, 2003; Dey et al., 2004; Komers et al., 2005; Xu et al., 2005) and a COX-2

inhibitor was found to ameliorate obesity-linked renal disease in this model (Dey et al., 2004). If COX enzymes can be altered by a specific CLA isomer or mixture of isomers, amelioration of early renal disease in the *fa/fa* Zucker rat could be achieved by dietary means.

Warford (2003) showed that a mixture of CLA isomers was able to ameliorate early renal disease progression in the *fa/fa* Zucker rat possibly via PPARs or modification of the eicosanoid enzyme $cPLA_2$. The question now is which CLA isomer is exerting the protective effects on the kidney. It is also of interest to determine whether the specific isomer/isomers/isomer mixture acts via modulation of rate limiting enzymes involved in eicosanoid production, namely $cPLA_2$, COX-1, COX-2.

HYPOTHESES

- Dietary CLA, specifically the *t*10, *c*12 isomer, will improve oral glucose tolerance in *fa/fa* Zucker rats as well as decrease body weight in this model of obesity and insulin-resistance.
- Early histological changes with regards to the MGVs in the kidney will be attenuated by the *t*10, *c*12 CLA isomer.
- As one of the proposed mechanisms for amelioration of early renal disease involves enzymes in eicosanoid production, feeding the *t*10, *c*12 CLA isomer will be associated with altered protein levels of COX-1, COX-2 and $cPLA_2$.

OBJECTIVES

1.) To examine the effects of specific CLA isomers on oral glucose tolerance and body weight of *fa/fa* vs. lean Zucker rats, and relationships with changes in the kidney parameters.

2.) To examine effects of specific CLA isomers on amelioration of histological changes in the size of the glomeruli of the kidney, via hematoxylin and eosin staining of kidney cross sections followed by measurement of MGV using microscopy.

3.) To examine the effects of specific isomers of CLA on steady-state protein levels of COX-1, COX-2 and $cPLA_2$ using western immunoblotting.

MATERIALS AND METHODS

fa/fa and Lean Zucker Rats

Male *fa/fa* and lean control Zucker rats were purchased from Harlan (Indianapolis, Indiana). The *fa/fa* Zucker rat was chosen for the study as this rat model displays obesity and insulin-resistance (Bray, 1977). It is also a good model for studying kidney disease associated with the metabolic syndrome (Toblli et al., 2004). Rats were 5 weeks of age upon arrival and were housed separately in hanging stainless steel cages and acclimatized for 5-7 days (n=80). Subsequently, rats were randomly assigned to a treatment diet (4 in total) for 8 weeks. A total of 8 experimental groups existed as each dietary treatment was fed to both *fa/fa* and lean Zucker rats, with n=10 per group.

Diet

The formulation of the diet was based on the AIN-93G diet, recommended to support growth (Reeves et al., 1993). **Table 3** shows the diet formulation. The diets contained 8.5% dietary fat consisting of soybean oil (Harlan Teklad, Madison, Wisconsin) and CLA (Natural ASA, Hovdebygda, Norway), or soybean oil alone (control). Diets were supplemented with 0.4% (4 g/Kg) CLA isomers singly or in combination. The 4 experimental diets were as follows: 1) 0.4% *c*9, *t*11 (Natural ASA), 2) 0.4% *t*10, *c*12 (Natural ASA), 3) 0.4% *c*9, *t*11 plus 0.4% *t*10, *c*12 (Natural ASA) and 4) control (no CLA). A pair-weighed group was included and fed the control diet in amounts that allowed for body weights equivalent to the CLA-isomer group with the lowest body weight, in order to

Table 3: Diet formulation

Ingredients [a]	**g/Kg diet**
Dry Mix	
cornstarch (Chefmate, Toronto, Ontario)	363
maltodextrin	132
sucrose	100
egg white	212.5
cellulose	50
AIN-93G mineral mix	35
AIN-93G vitamin mix	10
choline	2.5
biotin mix [b]	10
tert-butylhydroquinone [c]	0.014
Oil [d]	85

[a] All ingredients supplied by Harlan Teklad (Madison, Wisconsin) unless otherwise indicated

[b] biotin mix = 200 mg biotin/Kg cornstarch

[c] tert-butylhydroquinone supplied by Aldrich Chemical Company, Milwaukee, WI

[d] control diet = 85 g soy oil/Kg diet, CLA diet = soy oil plus enough CLA to give amounts defined in materials and methods section and to keep total oil amount at 85 g/Kg diet. Diets containing oil were stored at -20°C.

ensure differences in insulin sensitivity were not due to differences in body weight. This is different than having a pair-fed group where the pair-fed group of rats would have been given the exact amount of feed consumed by the diet group consuming the lowest amount of feed on the previous day. The pair-weighed group was not, however, included in the analysis of data as body weight did not differ significantly among diet groups. Feed batches were prepared in advance and stored at -20°C.

Feed cups were refilled with fresh feed three times per week (Monday, Wednesday and Friday) and feed consumption (adjusted for spillage) was recorded. All rats were fed ad libitum except for the pair-weighed groups that occasionally needed to be restricted. Pair-weighed rats that became restricted were fed daily in order to closely monitor weight changes and adjust feed amounts accordingly. Fresh water was provided to all rats ad libitum and was changed twice per week. Weekly body weights were recorded.

Oral Glucose Tolerance Testing (OGTT)

At 7.5 weeks of feeding, rats were subjected to an OGTT. One week prior to the actual test, rats went through a training session that involved fasting for 5 hours, restraint with a towel and consumption of a glucose solution. While restrained for practice, the back of an electric razor was held against the hind limb so that the rats could get used to the feel and sound of the razor, as the fur on the hind limb would have to be shaved on the test date for ease of blood collection. On the actual test date, a 70% glucose (Sigma, G7528) solution (70 g

glucose per 100 mL deionized water) was given at a dose of 1 g/Kg body weight immediately following a baseline blood sample (time=0). The solution was given orally with a plastic syringe and placed under the tongue of those rats not eager to drink it. Blood samples were taken at 0, 15, 30 and 60 minutes from the sephanous vein of the hind limb. The vein was pricked at a 90° angle with a 22 gauge needle (Becton Dickinson, Oakville, Ontario). One or both legs were used depending on vein visibility and ease of collection. Blood was collected in microvette tubes (Sarstedt, CB 300, 16.440.100, Numbrecht, Germany) and immediately placed on ice. Samples were spun in a microfuge (Eppendorf, Model No 5417C, Hamburg, Germany) at 11,000 x g for 10 minutes and serum aliquoted into 0.5 mL microcentrifuge tubes (Fisher Scientific, cat no 05-408-128, Nepean, Ontario).

Serum Glucose Determination

Commercial kits were purchased to determine serum glucose using a colorimetric assay (Diagnostic Chemicals Limited, cat no 220-32, Charlottetown, Prince Edward Island). The procedure involved a modified version of the Trinder glucose oxidase/peroxidase method developed by Trinder in 1969. The principle of the procedure is as follows:

glucose oxidase

glucose + O_2 + H_2O ⟶ gluconic acid + hydrogen peroxide (H_2O_2)

peroxidase

H_2O_2 + hydroxybenzoate + 4-aminoantipyrine ⟶ quinoneimine dye + H_2O

The intensity of color of the dye is proportional to the concentration of glucose in the serum sample.

Serum was diluted 5X with deionized water. A serial dilution of the glucose standard solution from the kit (5 mmol/L) was made with standards ranging in concentration from 0.625 mmol/L to 5 mmol/L. Sample, standard or blank (5 µL of each) were pipetted in triplicate into the wells of a 96-well microplate. Reconstituted glucose color reagent (200 µL) containing a buffer [pH=7.5, 0.25 mmol/L 4-aminoantipyrine, 20 mmol/L p-hydroxybenzoate, >40,000 U/L glucose oxidase (Aspergillus niger), >2000 U/L peroxidase (horseradish) and preservatives] was added to each well and the microplate was mixed gently. Microplates were incubated for 10 minutes at 18-26°C. Absorbance was read at 505 nm using a microplate reader (SpectraMax 340, Molecular Devices Corporation, Sunnyville, California) and SOFTmax Pro software was used to calculate glucose concentrations in the samples. The mean result was multiplied by 5 to obtain the final serum glucose concentration in

mmol/L. Samples with coefficients of variation (CVs) of 10 or higher were repeated.

Blood and Tissue Collection

After being on the experimental diets for 8 weeks, rats were fasted overnight (12 h) and killed the following morning by CO_2 gas asphyxiation and decapitation. Trunk blood was collected at this time for serum analysis. Various organs were dissected, weighed and placed in appropriate solutions for preservation. The kidneys were cleaned with a scalpel and tweezers, and weights were recorded. The right kidney was sliced longitudinally and half of the kidney was placed in a vial containing 10% phosphate-buffered formalin (PBF). After approximately 24 hours at room temperature, the half kidney was then transferred to phosphate-buffered saline (PBS) and stored at 4°C. The remaining one and a half kidneys were immediately wrapped in foil and frozen in liquid nitrogen. The foil containing the left kidney and half of the right kidney was then stored at -80°C.

Lyophilization of Kidneys

The left kidney from each rat was lyophilized in preparation for western immunoblotting. The left kidney was removed from the -80°C freezer and 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 with holes. The tube containing the cut up kidney was

weighed again and the initial weight of the frozen kidney was recorded. Tubes were then immersed in liquid nitrogen and placed in a pre-cooled (-40°C) lyophilizer (Labconco, Model No 4451 F, Kansas City, Missouri) so that samples could begin drying. All seals were greased so that a vacuum of less than 10 microns of mercury (Hg) was maintained. The freeze dryer was checked periodically to ensure the machine did not lose its seal. The tubes were removed from the freeze dryer and weighed every few hours or after an overnight drying period. When two consecutive equal tube weights were obtained, the freeze drying process was complete. If the weight was not equal to the last weight obtained, the tube was placed back into liquid nitrogen and was returned to the freeze dryer to continue drying. The final dry kidney weight was recorded. A spatula was used to pulverize the dried kidneys and a lid without holes was used to replace the porous lid. Samples were then stored at -80°C.

Homogenization of Kidneys

Cytosolic and particulate buffers (See **Tables 4** and **5** respectively for recipes) were prepared by first mixing all ingredients and then adding 5 M sodium hydroxide (NaOH salt, Fisher Scientific, s318-500, Napean, Ontario) to the solution to obtain a final pH of 7.2-7.4. Cytosolic buffer (2 mL or 100 volumes) was added to 20 mg of lyophilized kidney tissue in a glass, round-bottomed tube submerged on ice. Kidney tissue was homogenized for 2 x 30 seconds with a Polytron homogenizer (Brinkmann Instruments, Type PT 10 20 350D, Rexdale, Ontario) on a speed control setting of 5. Tubes were kept on ice

Table 4: Cytosolic buffer recipe for homogenization of kidney tissue in preparation for western immunoblotting

Ingredient [conc]	Amount	Final [conc]
500 mM tris-HCL (6.1 g/100 mL) Fisher Scientific, BP154-1, Napean, Ontario	2 mL	50 mM
0.5 M sucrose (17.2 g/100 mL) Sigma, S9378, Oakville, Ontario	10 mL	250 mM
200 mM EDTA (pH=7.6, 0.76 g/10 mL) Sigma, ED4SS	200 µL	2 mM
100 mM EGTA (pH=7.5, 0.38 g/10 mL) Sigma, E4378 Add NaOH to get it to dissolve and bring pH to 7.5	200 µL	1 mM
0.4 M NaF (0.168 g/10 mL) Sigma, S6521	2.5 µL	50 µM
deionized water	6.104 mL	
10 mM Na orthovanadate (1.839 mg/mL) Sigma, S6508	200 µL	100 µM
aprotinin (2.5 mg/mL) Sigma, A1153	200 µL	25 µg/mL
leupeptin (1 mg/mL) Sigma, L2884	500 µL	25 µg/mL
pepstatin (2.5 mg/mL, in 90:10 methanol:glacial acetic acid) Sigma, P5318	200 µL	25 µg/mL
soybean trypsin inhibitor (STI) (1 mg/mL) Sigma, T9003	20 µl	1 µg/ml
50 mM 4-(2-aminoethyl)benzene- sulfonyl fluoride (ABSF) (12 mg/mL) Sigma, A8456	57.6 µL	144 µM
β-mercaptoethanol Fisher Scientific, BP176-100	14 µL	10 mM
	Total 20 mL	

Table 5: Particulate buffer recipe for homogenization of kidney tissue in preparation for western immunoblotting

Ingredient [conc]	Amount	Final [conc]
500 mM tris-HCL (6.1 g/100 mL) Fisher Scientific, BP154-1, Napean, Ontario	2 mL	50 mM
0.5 M sucrose (17.2 g/100 mL) Sigma, S9378, Oakville, Ontario	10 mL	250 mM
200 mM EDTA (pH=7.6, 0.76 g/10 mL) Sigma, ED4SS	200 µL	2 mM
100 mM EGTA (pH=7.5, 0.38 g/10 mL) Sigma, E4378 Add NaOH to get it to dissolve and bring pH to 7.5	200 µL	1 mM
0.4 M NaF (0.168 g/10 mL) Sigma, S6521	2.5 µL	50 µM
deionized water	6.104 mL	
10% Triton X-100 Sigma, T8787	2 mL	1%
10 mM Na orthovanadate (1.839 mg/mL) Sigma, S6508	200 µL	100 µM
aprotinin (2.5 mg/mL) Sigma, A1153	200 µL	25 µg/mL
leupeptin (1 mg/mL) Sigma, L2884	500 µL	25 µg/mL
pepstatin (2.5 mg/mL, in 90:10 methanol:glacial acetic acid) Sigma, P5318	200 µL	25 µg/ml
soybean trypsin inhibitor (STI) (1 mg/mL) Sigma, T9003	20 µL	1 µg/mL
50 mM 4-(2-aminoethyl)benzene- sulfonyl fluoride (ABSF) (12 mg/mL) Sigma, A8456	57.6 µL	144 µM
β -mercaptoethanol Fisher Scientific, BP176-100	14 µL	10 mM
	Total 20mL	

at all times. Homogenate was poured into ultracentrifuge tubes and balanced before being inserted into a cold rotor (Beckman Coulter, Inc., model no 50.3TI, Fullerton, California) and placed in the pre-cooled (4°C) ultracentrifuge (Beckman, model no L8-80). Samples were spun at 100,000 x g for 35 minutes. This precipitated the particulate fraction while the cytosolic fraction remained in the supernatant. A transfer pipette was used to draw off the supernatant and this cytosolic fraction was placed into pre-labeled 2 mL microcentrifuge tubes (Fisher Scientific, cat no 05-408-141, Napean, Ontario) and stored at -80°C. The pellet was re-suspended in 0.4 mL (20 volumes) of particulate homogenization buffer. The tube was vortexed with a glass rod placed in it to help disperse the pellet in the buffer. After sitting on ice for 10 minutes, it was vortexed a second time. Tubes were once again balanced and spun at 100,000 x g for 35 minutes. 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 stored at –80°C.

Total Protein Determination

Total protein was determined in cytosolic and particulate fractions using the Bradford Method established in 1976. In this method, protein forms a complex with the dye brilliant blue G. The absorption maximum of the dye then shifts from 465 to 595 nm. The amount of absorption is proportional to the total amount of protein present in the sample.

A 96-well microplate was used, and wells were identified as blank, standard or sample. Standards were made ranging in concentration 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, and 10 µL of blank, standard or diluted sample was added to identified wells in triplicate. Bradford reagent (200 µL, Sigma, B6916), was added to each well using a multi-channel Eppendorf pipette. The plate was covered with a box and mixed on an orbital shaker (Fisher Scientific, model no 361 Nepean, Ontario) for 15 minutes at room temperature. The plate was read at a wavelength of 595 nm using a microplate reader (Molecular Devices Corporation, SpectraMax 340, Sunnyville, California). SOFTmax Pro software was used to calculate total protein concentrations in the samples. Mean values were multiplied by 20 to obtain the total protein concentration in mg/mL.

Protein Separation by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

As has been previously described (Aukema et al., 2002), one-dimensional sodium dodecyl sulfate (SDS) gel electrophoresis was used to separate proteins of interest, namely $cPLA_2$, COX-1 and COX-2. In this method, protein separation occurs as proteins migrate in an electrical field through a porous gel. Proteins will migrate down to different levels of the gel depending on their size, shape and charge. The pore size of the gel is also an important determinant in how the proteins will migrate.

Two glass plates were cleaned with methanol and placed with their cleaned sides together, with spacers separating the 2 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). Deionized water was poured in between the 2 plates to ensure there were no leaks. The water was then poured out and a 7.5% separating gel solution containing 2670 µL of deionized water, 1250 µL of 1.5 M tris (hydroxymethyl) aminomethane-hydrochloric acid (tris-HCL) pH 8.8 (tris base, Fisher Scientific, BP154-1, Napean, Ontario and HCL, Fisher Scientific, A144 500), 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) was made and added to the space in between the glass plates using a Pasteur pipette until it reached about ¾ of the way up the plates. Deionized water was then carefully layered on top of the separating gel, and the gel was left to polymerize for 30 minutes. The water was poured off and a 4% stacking gel was then prepared and added on top of the separating gel once again using a Pasteur pipette. The stacking gel solution contained 1625 µL of deionized water, 625 µL of 0.5 M tris-HCL pH 6.8, 25 µL of 10% (w/v) SDS, 250 µL of 40% bis-acrylamide, 12.5 µL of 10% APS and 2.5 µL of TEMED. A comb was inserted into the stacking gel in order to form lanes in which to load the protein samples. The gel was left to polymerize for 45 minutes. The bottom of the gel module was then unclipped

and placed in a chamber filled with used running buffer. Running buffer could be re-used approximately 10 times. The running buffer 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 was then diluted 10X with deionized water before use. New running buffer was poured into the upper compartment of the gel module and the comb was removed. The wells were washed with 100 µL of new running buffer using an Eppendorf pipette.

The amounts of total protein to be loaded for cytosolic $cPLA_2$, particulate $cPLA_2$ and COX-2 (14 µg of each) were based on dose-response curves (see **Figures 2** through **4,** respectively) and previous data from our lab (Warford, 2003). A slightly higher amount of COX-1 (20 µg) needed to be loaded as the kidney homogenates seemed to have low levels of this particular protein. Volumes of protein samples to be loaded were calculated based on the total kidney protein concentration determined by the Bradford method. The equation used was as follows for cytosolic $cPLA_2$, particulate $cPLA_2$ and COX-2 to load 14 µg of protein: 14 µL/protein concentration (µg/µL) = volume of sample (µL) and 7 µL - volume of sample (µL) = volume of deionized water (µL). Seven µL of Laemmli 2X sample buffer was added to the protein samples. The 2X sample buffer contained 3800 µL of deionized water, 1000 µL of 0.5 M tris-HCL pH 6.8 (tris base, Fisher Scientific, BP154-1, Napean, Ontario and HCL, Fisher Scientific, A144 500), 800 µL of glycerol (Fisher Scientific, BP229-1), 1600 µL of 10% (w/v) SDS (Fisher Scientific, BP166-100), 400 µL of 2-mercaptoethanol

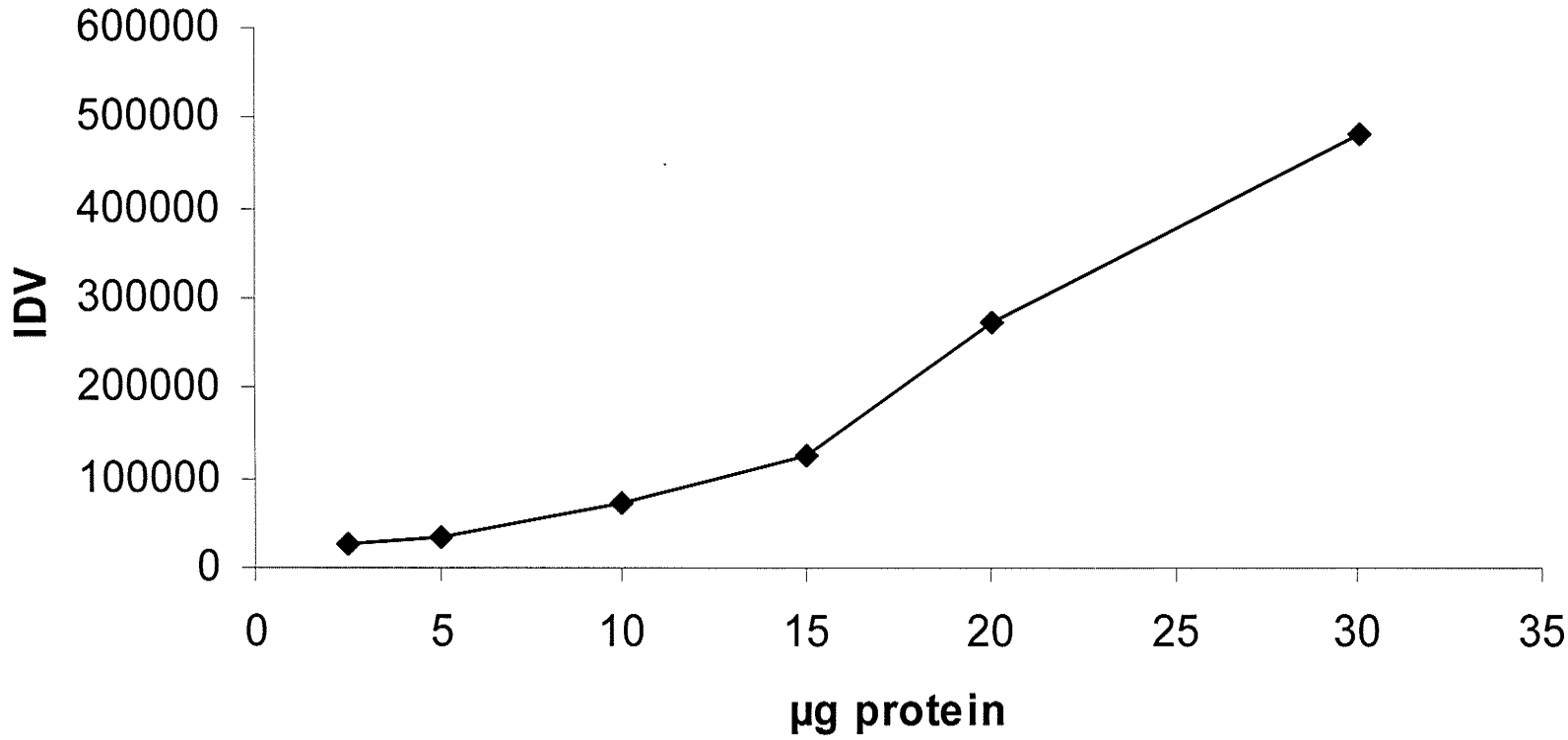


Figure 2: Dose-response curve for cytosolic $cPLA_2$

IDV = integrated density volume

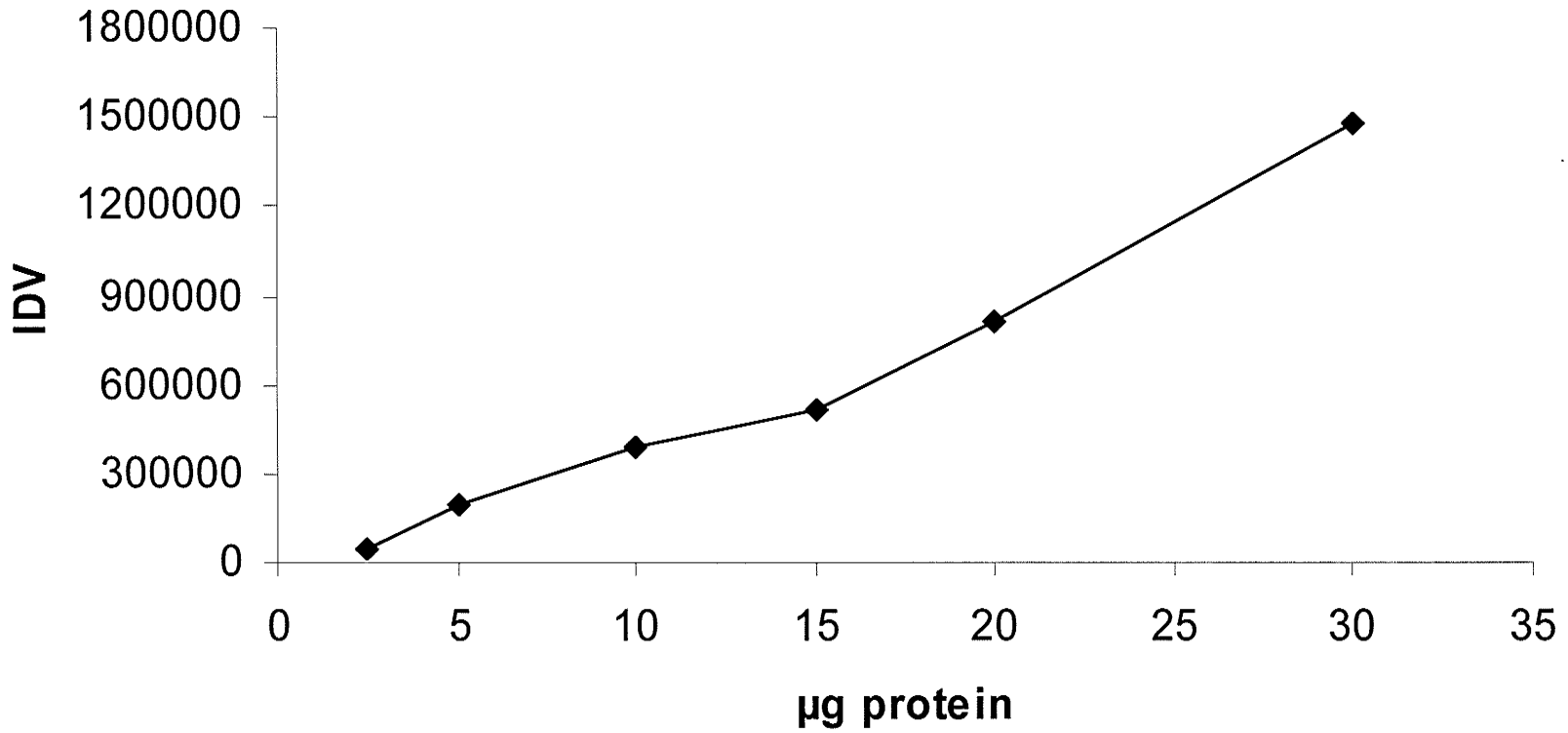


Figure 3: Dose-response curve for particulate $cPLA_2$

IDV = integrated density volume

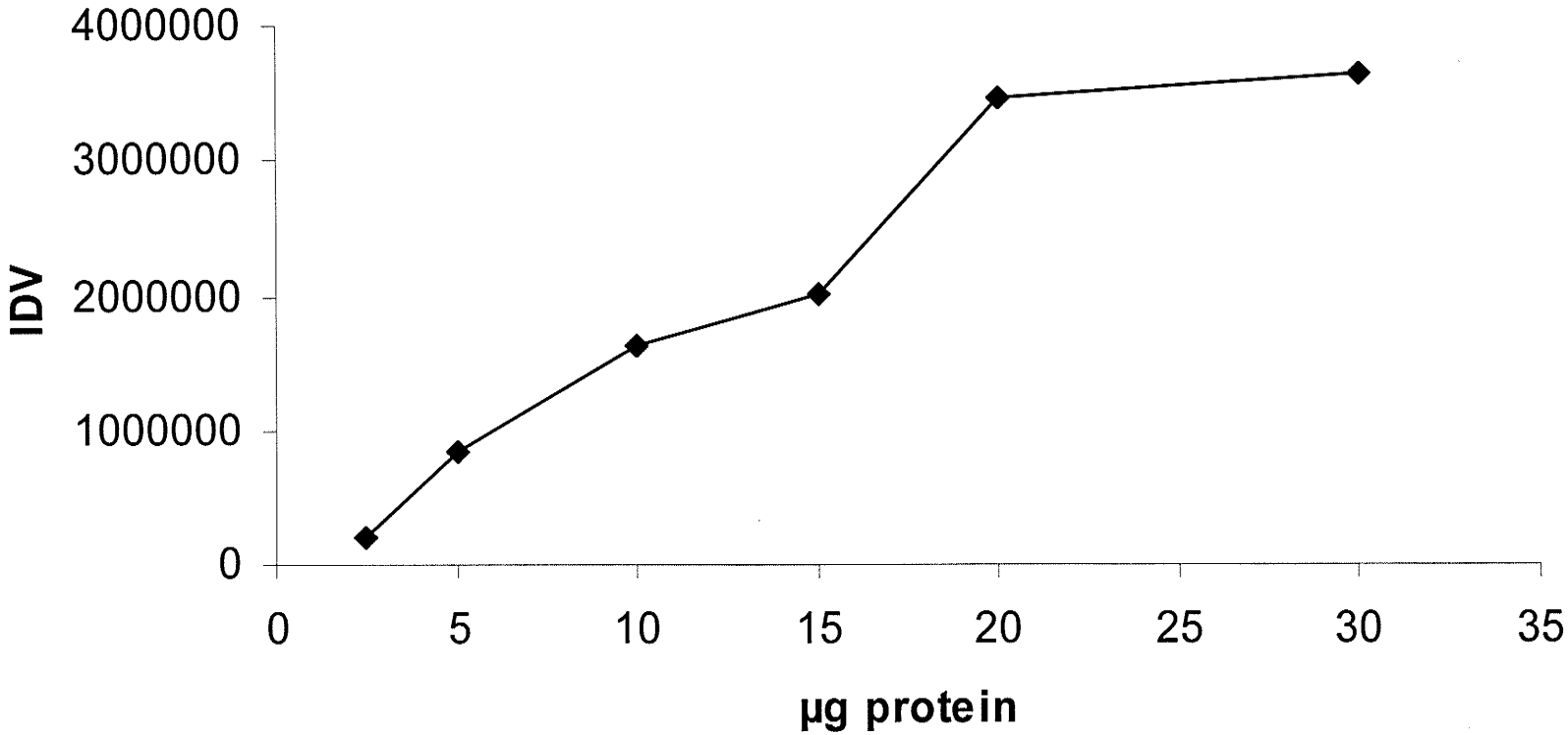


Figure 4: Dose-response curve for COX-2

IDV = integrated density volume

(Fisher Scientific, BP176-100) and 400 µL of 1% (w/v) bromophenol blue (Sigma, B-8026, Oakville, Ontario). A total of 20 µg of protein was loaded for COX-1. In order to calculate the amount of sample needed to load 20 µg of protein, a similar calculation to the one above was carried out. When the total protein concentration was less than 2.0 mg/mL, 6X sample buffer was used. Samples were placed on a heating block (Fisher Scientific, 11-718-2) at 90-100°C for 5 minutes to denature proteins. Samples were cooled on ice for 2 minutes, centrifuged (Eppendorf, Model No 5417C, Hamburg, Germany) at 5000 x g for 1 minute and placed back on ice before being loaded onto the gel.

The protein samples were loaded into the lanes of each gel along with a benchmark pre-stained protein ladder (Invitrogen, cat no 10748-010, Burlington, Ontario) and a standard run in duplicate. The standard was prepared by combining 50 µL of particulate sample from 32 rats (4 rats from each group). The proteins migrated in running buffer in an electrophoretic chamber (Amersham Biosciences, part of Hoefer miniVE vertical electrophoresis system, Amersham Biosciences, 80-6418-77, Buckinghamshire, England) at 200V at room temperature until the dye front just came off the gel (approximately 1 hour and 50 minutes).

Western Immunoblotting

Before the dye front ran off the gels, a hydrophobic polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Hybond-P RPN303F, Buckinghamshire, England) was cut to the proper size and labeled. The lower

left hand corner of the membrane was cut to indicate the first lane. 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 until the gel was ready for transfer. The transfer buffer was made as 10X transfer buffer and 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. Before use, 100 mL of 10X transfer buffer was mixed with 200 mL methanol (Fisher Scientific, A452-4) and 700 mL deionized water. When the gel was finished running, the gel module was removed from the chamber and the glass plates were separated in order to access the gel. The stacking gel was cut off using the spacer and a small amount was cut off from the bottom of the gel. The bottom corner also was cut off to indicate lane number 1. The gel was then equilibrated in transfer buffer for 5-10 minutes.

The gel was then placed in a blot module (Amersham Biosciences, 80-6418-96, Buckinghamshire, England) against the PVDF membrane, in between two pieces of filter paper with the cut corners matching up and oriented in the upper right hand corner of the apparatus. A sponge was placed on one side of the gel/membrane, and enough sponges were placed on the other side to form a tight fit in the "sandwich". Everything was kept wet with used transfer buffer. Transfer buffer could be reused up to 10 times. The transfer apparatus was placed in a chamber filled with used transfer buffer, and new transfer buffer was

added to the upper compartment of the transfer apparatus. The apparatus was connected to the power unit and transferred in the fridge (4°C) for 2 hours at 375 milliamps (mA).

After the transfer, the membrane was placed on a piece of filter paper and cut horizontally with a clean scalpel in between the 2 proteins of interest, using the benchmark ladder as a guide. This was only done if the membrane needed to be blotted with 2 different primary antibodies. The membrane was then "blocked" in order to help minimize non-specific binding of the antibodies. The membrane was placed in a dish and lines from the benchmark ladder were marked with pen to ensure that they remained visible throughout the rest of the immunoblotting procedure. The membrane was then placed on a rocker (Boekel Scientific, rocker II model 260350, Feasterville, Pennsylvania) for 1 hour in a 5% skim milk solution (0.5 g skim milk powder in 10 mL 0.1% tris base solution (TBS)/Tween). The TBS/Tween was made as 10X TBS/Tween from 24.2 g tris base (Fisher Scientific, BP154-1, Napean, Ontario) and 80 g sodium chloride (NaCl, Fisher Scientific, BP358-212). This was made up to 800 mL with deionized water. The pH was then adjusted to 7.6 using HCl. Ten mL of Tween (Fisher Scientific, BP337-500) was then added to make a 1% Tween solution. This solution was then diluted 10X with deionized water to give a final Tween concentration of 0.1%. After the 1 hour of blocking, the skim milk solution was poured off the membrane so it could be covered with the primary antibody solution.

The primary antibody was prepared in 2% skim milk solution (0.2 g skim milk powder in 10 mL TBS/Tween). The primary antibody concentration was 1:250 for all proteins, namely $cPLA_2$ [Santa Cruz Biotechnology, N-216, anti-human rabbit polyclonal immunoglobulin G (IgG), cat no sc-438, Santa Cruz, 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). The primary antibody solution was poured onto the membrane and the membrane was covered and placed on a rocker (Sanyo GallenKamp, cat no IH-370, APP IB1684, Loughborough, Leicestershire, England) in the fridge at 4°C overnight. The next morning the primary antibody was removed and saved in the -20°C freezer. It could be reused up to 5 times. The membrane was then washed 3 times in TBS/Tween by being placed on the orbital shaker (Fisher Scientific, Model No 361, Napean, Ontario) at 90 rpm for a specific length of time that depended on the protein of interest. It was washed for 3 x 5 minutes (total 15 minutes) for cytosolic and particulate $cPLA_2$, and for 3 x 10 minutes (total 30 minutes) for COX-1 and COX-2. The secondary antibody was then prepared in TBS/Tween and added to the membrane. For cytosolic and particulate $cPLA_2$, as well as for COX-2, anti-rabbit IgG was used at a concentration of 1:20,000 (Sigma, cat no A-0545). For COX-1, anti-mouse IgG was used at a concentration of 1:20,000 (Sigma, cat no A-3682). The membrane containing the secondary antibody solution was then placed on a rocker (Boekel Scientific, rocker II model 260350, Feasterville, Pennsylvania) for 1 hour at room

temperature. The membrane was washed a second time with TBS/Tween on the orbital shaker (Fisher Scientific, Model No 361) set at 90 rpm. For each protein of interest, the length of time for the second wash was the same as for the first wash. The membrane was then ready to be developed.

Two chemiluminescent substrates (Fisher Scientific, ChemiGlow cat no 2900811, Napean Ontario) were mixed with deionized water in a 1:1:4 ratio 4-5 minutes before it was needed. The membrane was then placed on a piece of plastic wrap and the chemiluminescent mixture was evenly distributed over the entire membrane using a Pasteur pipette. This was left to sit for 5 minutes. The liquid was then drained off and the membrane placed on the top portion of a new piece of plastic wrap. The bottom half of the wrap was then carefully folded over the membrane, so as not to trap any air bubbles. The excess plastic wrap was cut using a scalpel and any air bubbles were removed. The membrane was developed in an imaging system (Alpha Innotech, San Leandro, California) and analyzed using appropriate computer software (AlphaEase FC, version 3.1.2 and 4.0.0).

The integrated density volume (IDV) was calculated and used as a measure of the intensity of the protein band in pixels on the computer screen. In order to obtain the IDV, an object box was placed around the protein band, large enough to encompass the entire band, but no larger than necessary. A background box was then placed underneath the band and as close to the band as possible, to get a representative section of the background staining surrounding the protein band. These 2 boxes were then linked together so that

the background pixels could be subtracted from the protein band pixels, in order to obtain the IDV. The IDV is representative of the amount of the particular protein of interest that was present in the sample. The IDV of the protein sample was then divided by the average IDV of the 2 standards in order to control for gel to gel variation. The IDV was expressed in arbitrary units.

Urine Collection

Rats were placed in metabolic cages for 12 hours overnight in order to obtain a timed urine collection. They were given water, but no feed. Pre-weighed urine collection vials were taped to the bottom of the collection funnels in the metabolic cages. In the morning, the urine collection vials were removed from the metabolic cages and weighed a second time and the urine weight recorded. Aliquots of urine were transferred with a pipette into labeled 1.5 mL microcentrifuge tubes (Fisher Scientific, cat no 05-408-129, Napean, Ontario) and stored at -80°C.

Urinary Protein

Urinary protein was determined by a colorimetric assay, based on the method described by Smith et al. (1985). This assay is based on the principle that protein will form a complex with copper II (Cu^{2+}), which will then be reduced to copper I (Cu^{+}). The Cu^{+} will then form a complex with bicinchoninic acid (BCA), which appears purple-blue in color. The absorbance of this color is proportional to the total protein present.

Fifty µL of a 20% ice-cold trichloroacetic acid (TCA) solution (Fisher Scientific, A322-500, Napean, Ontario) was added to 50 µL of urine in a 1.5 µL microcentrifuge tube in order to precipitate the proteins (Fisher Scientific, 05-408-129, Napean, Ontario). Samples were vortexed and then placed into the microfuge (IEC, MicroMax) for 5 minutes at 21,000 x g. The supernatant fraction was removed and discarded. The protein pellet was then re-suspended in the tube with 100 µL PBS and vortexed. Each unknown was diluted to a ratio of 1:5 with PBS (10 µL of re-suspended protein with 40 µL of PBS). Standards were made using a BSA stock solution (2 mg/mL) and ranged in concentration from 0.125 mg/mL to 2 mg/mL. Blank, standards and samples (10 µL) were added to wells of a 96-well microplate in triplicate. The reagent was a 1:50 dilution of copper II sulfate ($CuSO_4$, Sigma, C2284, Oakville, Ontario) to BCA (Sigma, B9643). $CuSO_4$ was added to the BCA immediately before it was time for the reagent to be added to the wells. The reagent was mixed and then 200 µL was added to each well. The plate was left to incubate for 30 minutes at 37°C. The absorbance was read at 562 nm using a microplate reader (Molecular Devices Corporation, SpectraMax 340, Sunnyville, California). The protein concentrations of the urine samples were calculated using SOFTmax Pro software.

Urinary Creatinine

Urinary creatinine was determined using a colorimetric assay, based on the principle developed by Heinegard and Tiderstrom in 1973. When creatinine is mixed with alkaline picrate, a yellow/orange color will result. When treated with

an acidic solution, the creatinine-picrate color will fade faster than the color of other interfering substances that formed color complexes with picrate. The amount of creatinine is therefore proportional to the difference in color intensity measured at 500 nm, before and after adding an acidic solution.

Standards (Diagnostic Chemicals Limited, Charlottetown, Prince Edward Island) were made ranging in concentration from 1 mg/dL to 4 mg/dL. Urine samples were diluted 20X with deionized water. Standard, diluted sample or blank (100 µL) of each was mixed with 1 mL of a picric acid solution in a 1 mL cuvette. The picric acid solution contained a 0.05 M sodium phosphate ($Na_2HPO_47H_2O$) and 0.05 M sodium borate ($BNaO_24H_2O$) solution ($Na_2HPO_47H_2O$, Fisher Scientific, BP330-500, Napean, Ontario and $BNaO_24H_2O$, Sigma, s0251, Oakville, Ontario), which was made by mixing 13.4 g $Na_2HPO_47H_2O$ and 6.9 g of $BNaO_24H_2O$ with 900 mL deionized water. NaOH pellets (Fisher Scientific, S318-500) were then added until the solution reached a pH of 12.7. The solution was then made up to 1 L with deionized water. The picric acid solution was made by mixing 2 volumes of the 0.05 M $Na_2HPO_47H_2O$ and 0.05 M $BNaO_24H_2O$ 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). Once the picric acid solution was added to the cuvettes, the standards and samples were left to incubate for 45 minutes at room temperature. The absorbance of each cuvette was then read at a wavelength of 500 nm using a spectrophotometer (Milton Roy, Spectronic 3000 Array). Following the reading, 25 µL of a 60% acetic acid (Fisher Scientific, A38-212) solution was added to

each cuvette. The standards and samples were then left to incubate for 6 minutes at room temperature and then read a second time 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 constructed in Microsoft Excel based on the final absorbance of each concentration of standard, and the concentration of creatinine in the urine samples was calculated using the equation from this standard curve.

Urinary Glucose

Commercial kits were purchased to determine urinary glucose using a colorimetric assay (Diagnostic Chemicals Limited, cat no 220-32, Charlottetown, Prince Edward Island). The principle and procedures of the assay are described under *Serum Glucose Determination* above. The changes to the assay are noted below.

Serial dilutions of the stock glucose standard from the kit (5 mmol/L) were made up and ranged in concentration from 0.313 mmol/L to 5 mmol/L. Urine samples that were out of range were diluted accordingly and re-analyzed.

Serum Creatinine

Serum creatinine was determined using a colorimetric assay. The principle of the assay is described above under *Urinary Creatinine* and the modifications of the procedure are noted here.

Serum was spun in the centrifuge (Eppendorf, Model No 5417C, Hamburg, Germany) at 11,000 g for 6 minutes so that any lipid in the serum would come to the top. An Eppendorf pipette was then passed through the lipid to the bottom of the tube, so that the serum could be 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) and ranged in concentration from 0.5 mg/dL to 10 mg/dL. Blank, standard, or serum sample (20 µL) were added in triplicate to wells of a 96-well microplate. Picric solution (200 µL) was added to each well using a multi-channel Eppendorf pipette. All air bubbles were popped with a needle. The plate was placed in the microplate reader (Molecular Devices Corporation, SpectraMax 340, Sunnyville, California) and left to incubate for 45 minutes at room temperature. The plate was mixed in the microplate reader at the beginning, in the middle and at the end of the incubation time. Absorbance was read at 500 nm. Twenty µL of a 15% acetic acid solution was then added to each well using a multi-channel Eppendorf pipette. The 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 that was made daily. The plate was once again placed into the microplate reader and left to incubate for 6 minutes. It was once again mixed while in the microplate reader. The plate was read again at 500 nm. The final absorbance was calculated as follows: final absorbance = pre-acid absorbance – post-acid absorbance. A standard curve was once again constructed in Microsoft Excel

based on the final absorbance of each concentration of standard, and the concentration of creatinine in the serum samples were calculated using the equation from this standard curve. Creatinine clearance was then calculated using the equation:

$$\frac{\text{concentration of creatinine in urine (mg/dL) x volume urine (mL)/time (min)}}{\text{concentration of creatinine in serum (mg/dL)}}$$

Mean Glomerular Volume

i) Sectioning and Staining of Kidneys

Kidneys were removed from PBS and placed in PBF. Paraffin blocks were prepared. They were sectioned at 5 microns using a Microtome (American Optical 820, Southbridge, Massachusetts). Kidney sections were placed in xylene to remove the paraffin and stained with hematoxylin and eosin. The hematoxylin stains nuclei and ribosomes blue while the eosin stains the kidney proteins pink. First, the kidney sections were stained with Harris's hematoxylin for 5 minutes, washed with deionized water, placed in 1% HCL in deionized water and then placed in 2% ammonia water. The kidney sections were then washed with deionized water, stained with eosin for 3 minutes, and dehydrated with alcohol. Cover slips were mounted with cytoseal.

ii) Capturing Glomeruli using Microscopy

Stained slides of longitudinal kidney cross sections were first blinded to remove potential bias during analysis by microscopy. Pictures of glomeruli (30 per slide) 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 the 20X magnification lens. The pictures were taken sequentially using an appropriate software program (Spot Advanced). Images that appeared out of focus were retaken in order to ensure that the edge of the glomerulus could be distinguished.

iii) Measuring Glomeruli

Captured images of glomeruli were measured using the Image Pro Plus 2 software program. A 20X calibration grid was first opened, and then the file to be analyzed was opened over top of the 20X calibration grid. The largest diameter across each whole glomerulus in the picture was measured. Since there were 30 images for each rat, this ensured there were a minimum of 30 glomeruli measured per rat. The values for glomerular width (measured in μm) were exported into a Microsoft Excel file.

iv) Calculation of MGV

MGV was calculated based on the method first described by Weibel & Gomez (1962). In order to calculate MGV, an average glomerular diameter was first calculated for each rat. This number was then divided by 2 to give an

average radius for each animal. A MGA was then calculated for every rat using the formula πr^2 (the area of a sphere). Subsequently, MGV was calculated using the formula $MGV = 1.25 \times MGA^{3/2}$. The value 1.25 comes 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 (Hirose et al., 1982).

Statistical Analysis

Data were analyzed using the statistical software program SAS (SAS Institute Inc., version 9.1, Cary, North Carolina). Data was analyzed for normality using the Kolmogorov-Smirnov D Statistic and for homogeneity of variance using Levene's Test for Homogeneity of Variance. Data that was not normally distributed was Log transformed and if it continued to be not normal was ARCTAN transformed. If data still was not normal, outliers were removed if >3 standard deviations away from the mean. A 2-way analysis of variance (ANOVA) was used to analyze main effects (genotype and diet) and interactions. Pre-planned contrasts were used to determine significance for specific interactions. If interactions were present, then the data was analyzed by a 1-way ANOVA, followed by Duncan's Multiple Range test. Statistically significant pre-planned contrasts were reported in every case, regardless of whether or not an overall interaction existed in the ANOVA table. Duncan's Multiple Range Test was used to determine differences between means when diet, interaction (genotype x diet) or pre-planned contrasts were significant. Pre-planned contrasts gave the same interpretation of results as the Duncan's Multiple Range Test in all cases except for the parameters of total feed intake (**Figure 5**) and kidney weight/body weight

% (**Figure 8**). A P-value of <0.05 was considered statistically significant for main effects. A P-value of <0.10 was considered statistically significant for interactions as determined by the ANOVA table or pre-planned contrasts. A repeated measures program was used to analyze serum glucose (from the OGTT) over time. Non-transformed data are reported in the Results section as mean ± standard error of the mean (SEM).

Presentation of Data

Figures in the results section will show all 8 diet/genotype groups except for protein/creatinine ratio which needed to be graphed differently in order to show significant differences within the two main effects. The bars represent mean ± SEM (n=10 in most cases with exceptions noted). Diets are as described in the first figure in the results section (**Figure 5**). A 2-way ANOVA (2 genotypes and 4 diets) was used to test main effects and interactions. Pre-planned contrasts were used to determine significance for specific interactions. If interactions were present, then the data was analyzed by a 1-way ANOVA, followed by Duncan's Multiple Range test. If only genotype or diet effects existed, this will be indicated at the top right hand side of the graph near the legend. If an interaction existed, as indicated by either the 2-way ANOVA and/or a pre-planned contrast, then a 1-way ANOVA followed by the Duncan's Multiple Range Test was performed as post-hoc analysis and small letters above the bars showing significantly different means will be indicated on the graph. The P-

values for the interaction will be reported in the legend. The means ± SEM for all 8 diet/genotype groups are presented in tabular form in the appendix.

RESULTS

Feed Intake and Body Weight

As evidenced by **Figure 5**, there was a diet x genotype interaction (P=0.0126) from the ANOVA for total feed intake throughout the study in that the *fa/fa* rats on the 10,12 diet ate significantly less than *fa/fa* rats on all other diets. The pre-planned contrast for lean versus *fa/fa* for 10,12 versus CTL was in agreement with this (P=0.0049). Also, the *fa/fa* rats on the TOG diet ate significantly less than *fa/fa* rats on the CTL diet. The pre-planned contrast for lean versus *fa/fa* for TOG versus CTL was not in agreement with this (P=0.1245). The *fa/fa* rats had a greater total feed intake compared to the lean rats for all diets. For example, the *fa/fa* rats on the CTL diet ate an average of 1666 $\pm$ 51 g of feed and the lean rats on the CTL diet ate an average of 1022 $\pm$ 24 g of feed throughout the 8 week study period.

Despite the lower feed intake in the *fa/fa* 10,12 group, there was no effect of diet on body weight. At the end of the study, the *fa/fa* rats weighed 67% more than the lean rats (554 $\pm$ 7 g versus 331 $\pm$ 3 g, respectively, P<0.0001, **Figure 6**).

Kidney Weight

A pre-planned contrast was significant (P=0.067) for *fa/fa* rats on the 10,12 diet having a smaller kidney weight than *fa/fa* rats on the CTL diet and a similar interpretation was obtained with Duncan's Multiple Range Test (**Figure 7**). The *fa/fa* rats had a greater kidney weight compared to lean rats for all diets. For

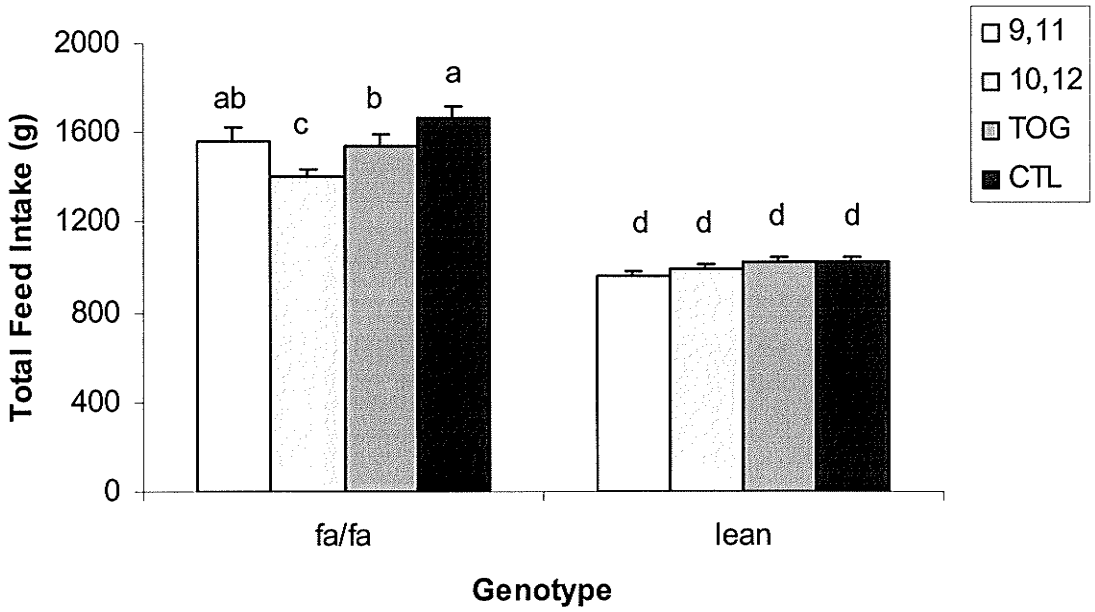


Figure 5 Total feed intake for *fa/fa* and lean Zucker rats at the end of the 8 week study. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets are as follows: 9,11 represents 0.4% of the *c*9, *t*11 CLA isomer, 10,12 represents 0.4% of the *t*10, *c*12 CLA isomer, TOG (together) represents 0.4% of the *c*9, *t*11 CLA isomer plus 0.4% of the *t*10, *c*12 CLA isomer and CTL represents the control diet (soybean oil with no CLA). A 2-way ANOVA (2 genotypes and 4 diets) was used to test main effects and interactions. Pre-planned contrasts were also used to test for interactions. If interactions were present, then the data were analyzed by a 1-way ANOVA, followed by Duncan's Multiple Range Test. Different letters indicate means are significantly different as determined by Duncan's Multiple Range Test. Significant genotype x diet interaction, P=0.0126 and significant CONTRAST lean versus *fa/fa* for 10,12 versus CTL, P=0.0049.

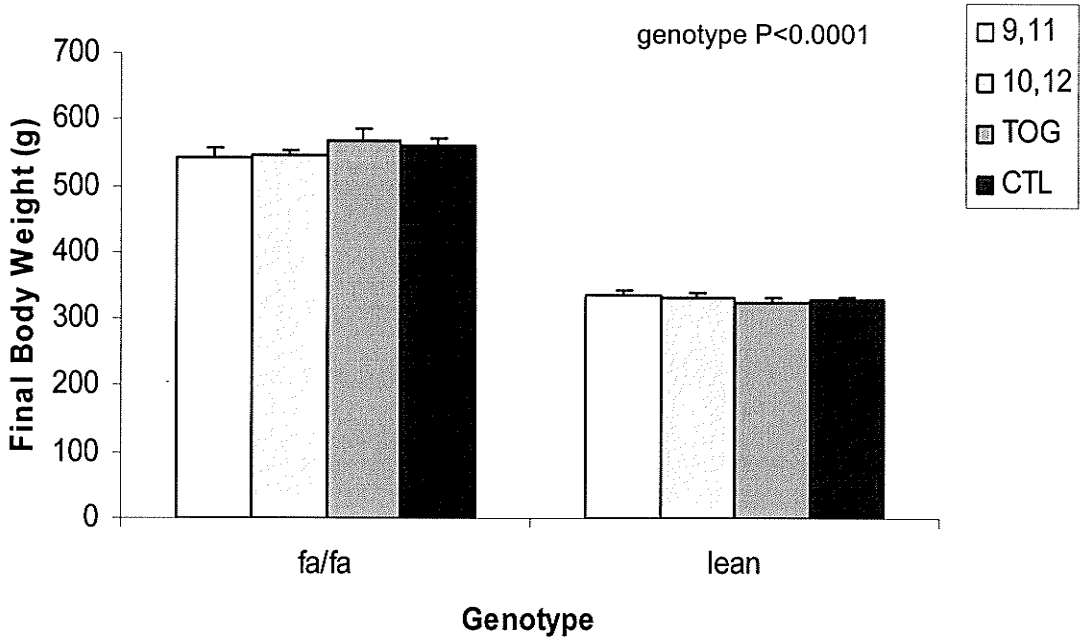


Figure 6 Final body weight of *fa/fa* and lean Zucker rats at the end of the 8 week study. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets and statistical analysis are as described in Figure 5.

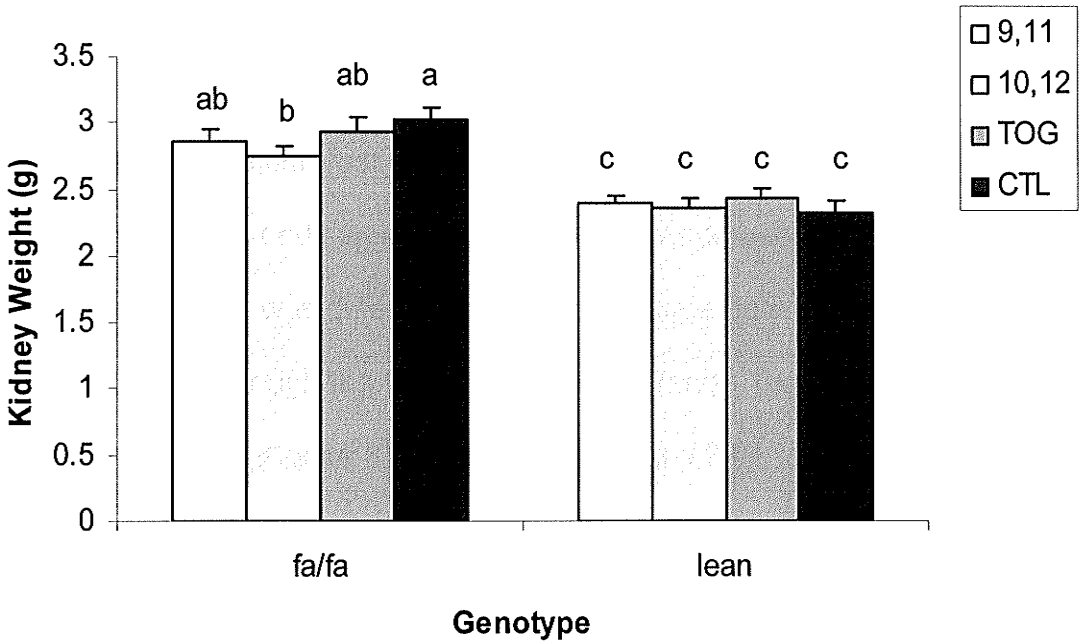


Figure 7 Kidney weight of *fa/fa* and lean Zucker rats at the end of the 8 week study. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets and statistical analysis are as described in Figure 5. Significant CONTRAST lean versus *fa/fa* for 10,12 versus CTL, P=0.067.

example, the *fa/fa* rats on the CTL diet had a kidney weight of 3.0 ± 0.1 g versus a kidney weight of 2.3 ± 0.1 g for lean rats on the CTL diet.

A pre-planned contrast was significant (P=0.0335) for kidney weight expressed per 100 g body weight in that the difference between lean and *fa/fa* rats for the TOG diet was different than the difference between lean and *fa/fa* rats for the CTL diet, although the Duncan's Multiple Range Test was not in agreement with this (**Figure 8**). The *fa/fa* rats had a 38% lower kidney weight per 100 g body weight compared to lean rats for all diets (0.52 ± 0.01% versus 0.72 ± 0.01%, respectively).

Urinary Parameters

Figure 9 demonstrates that the *fa/fa* rats had a 47% higher volume of urine excreted in 12 hours compared to the lean rats (6.6 ± 0.5 mL/12 h versus 4.5 ± 0.3 mL/12 h, respectively, P=0.0010).

Urine protein was 76% higher in *fa/fa* compared to lean rats (14.18 ± 1.26 mg/12 h versus 8.05 ± 0.81 mg/12 h, respectively, P=0.0002, **Figure 10**).

As shown in **Figure 11**, urinary creatinine was lower in *fa/fa* rats compared to lean rats (3.37 ± 0.19 mg/12 h versus 4.88 ± 0.20 mg/12 h, respectively, P<0.0001).

When urine protein was expressed per mg urine creatinine, the *fa/fa* rats had a higher urine protein/creatinine ratio compared to the lean rats as indicated by the ANOVA (4.35 ± 0.30 mg/mg versus 1.65 ± 0.15 mg/mg, respectively, P<0.0001, **Figure 12**). Diet was also significant (P=0.0447) as indicated by the

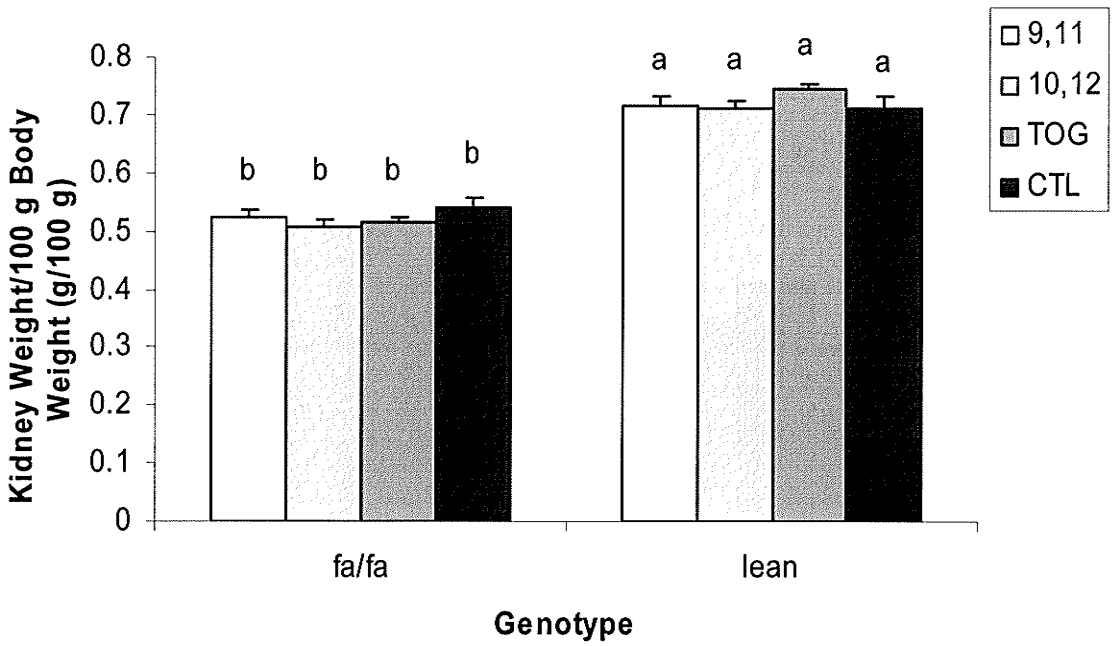


Figure 8 Kidney weight expressed per 100 g body weight in *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets and statistical analysis are as described in Figure 5. Significant CONTRAST lean versus *fa/fa* for TOG versus CTL, P=0.0335. There was no significant differences among diet groups based on means testing with Duncan's Multiple Range Test.

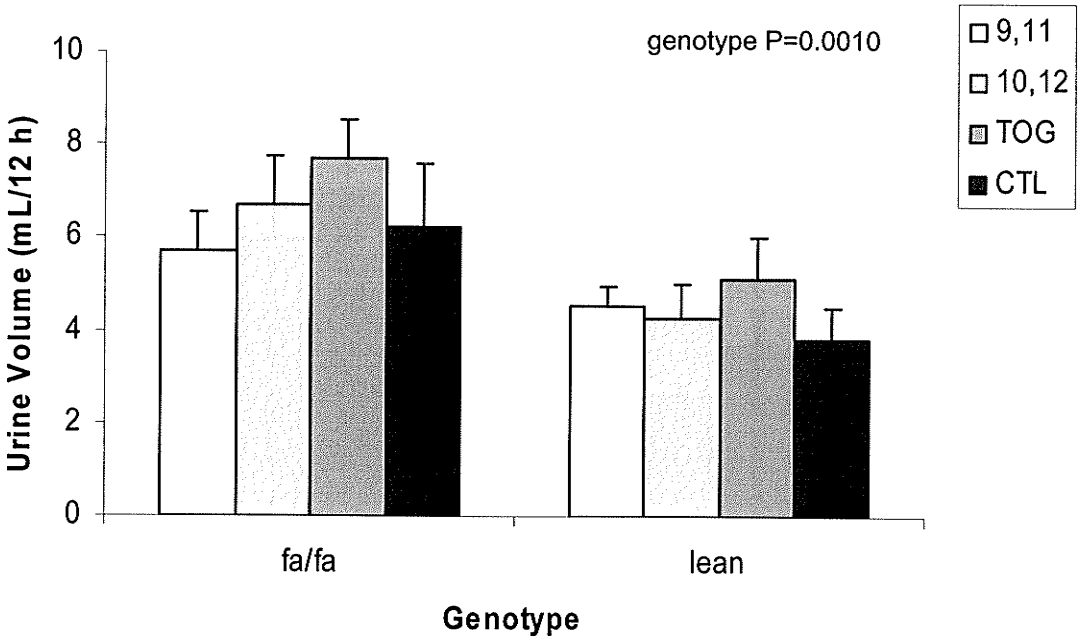


Figure 9 Urine volume excreted by *fa/fa* and lean Zucker rats in 12 hours. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets and statistical analysis are as described in Figure 5.

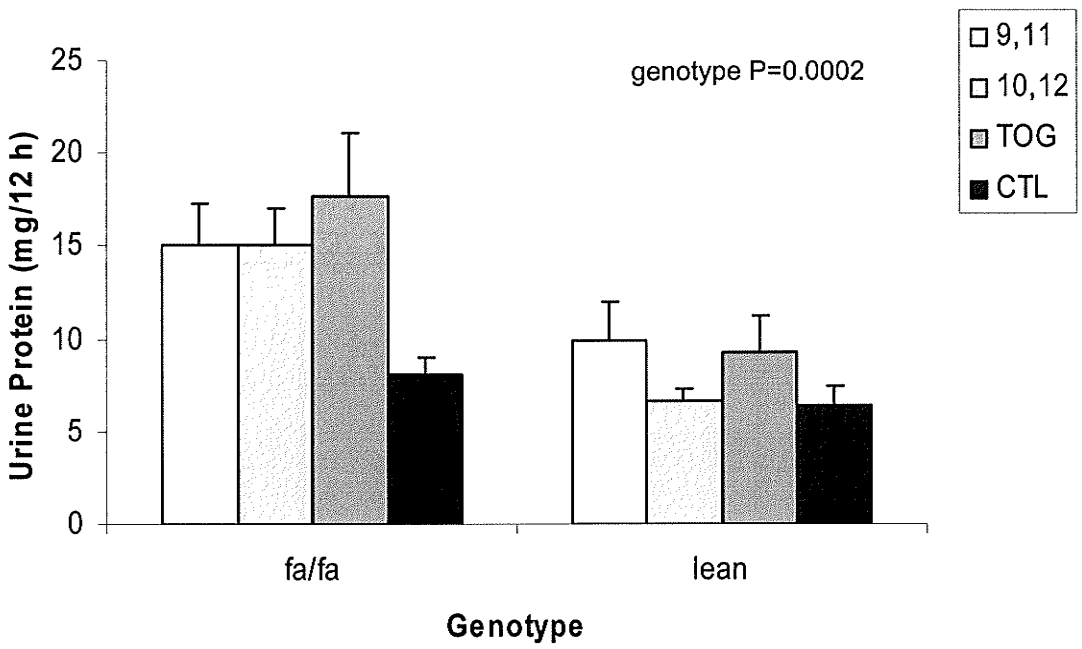


Figure 10 Urine protein excreted by *fa/fa* and lean Zucker rats in 12 hours. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10 except for *fa/fa* CTL where n=8 and *fa/fa* TOG where n=9). The four different diets and statistical analysis are as described in Figure 5.

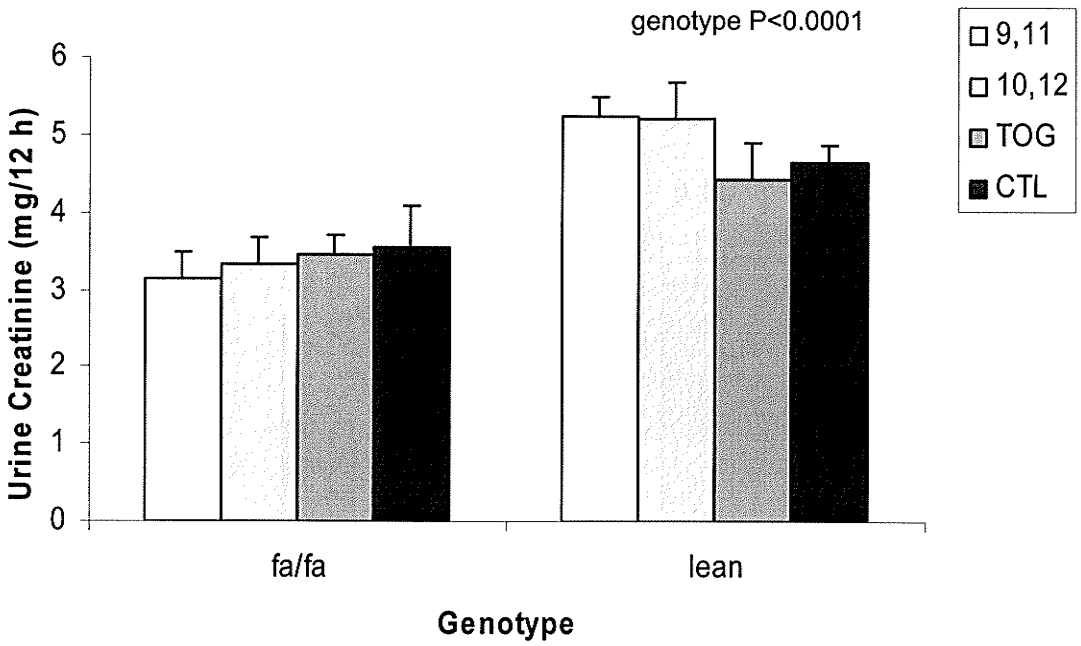


Figure 11 Urine creatinine excreted by *fa/fa* and lean Zucker rats in 12 hours. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets and statistical analysis are as described in Figure 5.

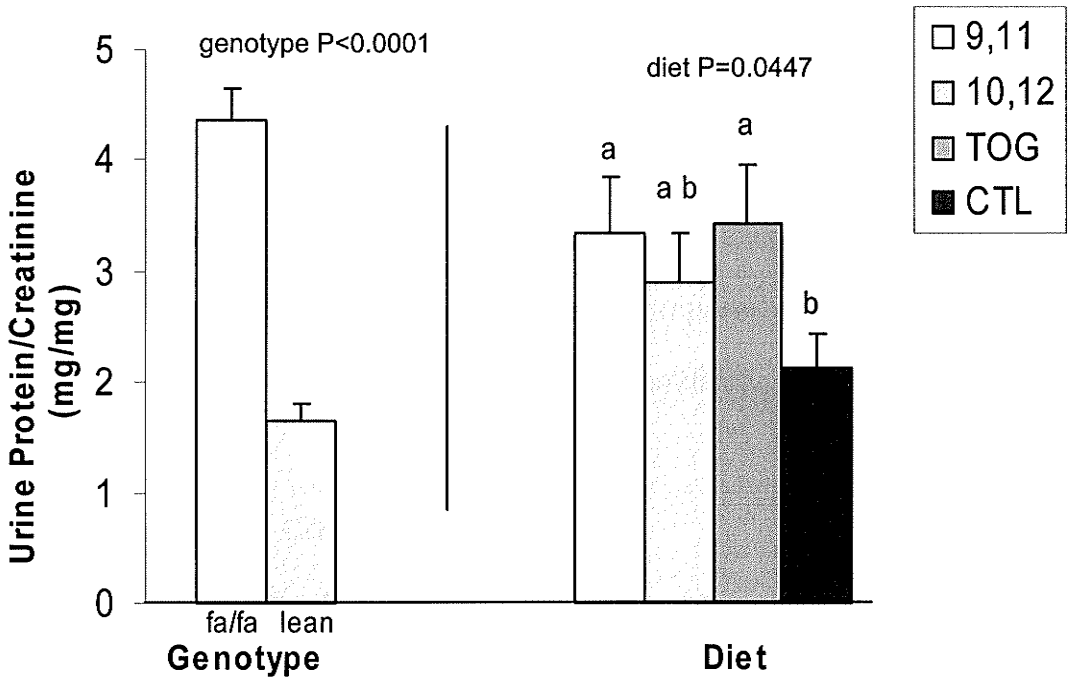


Figure 12 Urine protein expressed per urine creatinine for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for the 2 genotype groups (n=38 for *fa/fa* and n=40 for lean) and 4 diet groups (n=20 except for CTL where n=19 and TOG where n=19). The four different diets and statistical analysis are as described in Figure 5.

ANOVA in that urine protein/creatinine was significantly elevated in rats fed the 9,11 and TOG diets relative to the CTL diet, but was not significantly elevated in rats fed the 10,12 diet.

Figure 13 shows the amount of urinary glucose excreted in 12 hours. The *fa/fa* rats had a higher urinary glucose excretion compared to lean rats (7.00 ± 0.82 µmol/12 h versus 4.17 ± 0.22 µmol/12 h, respectively, P=0.0004).

Serum Creatinine and Creatinine Clearance

The *fa/fa* rats had a higher serum creatinine concentration compared to lean rats (44.64 ± 2.50 µmol/L versus 33.68 ± 1.72 µmol/L, respectively, P<0.0001, **Figure 14**).

Creatinine clearance was calculated, and the results are displayed in **Figure 15**. The *fa/fa* rats had a lower creatinine clearance than lean rats (0.97 ± 0.07 mL/min versus 1.90 ± 0.10 mL/min, respectively, P<0.0001).

Creatinine clearance was then adjusted to 100 g body weight. As evidenced by **Figure 16**, the *fa/fa* rats still displayed a lower creatinine clearance compared to the lean rats (0.18 ± 0.01 mL/min/100 g body weight versus 0.54 ± 0.02 mL/min/100 g body weight, respectively, P<0.0001).

Mean Glomerular Volume (MGV)

A pre-planned contrast was significant (P=0.0624) for MGV showing *fa/fa* rats on the 10,12 diet having a smaller MGV than the *fa/fa* rats on the CTL diet, **Figure 17**. The Duncan's Multiple Range Test was in agreement with this. The

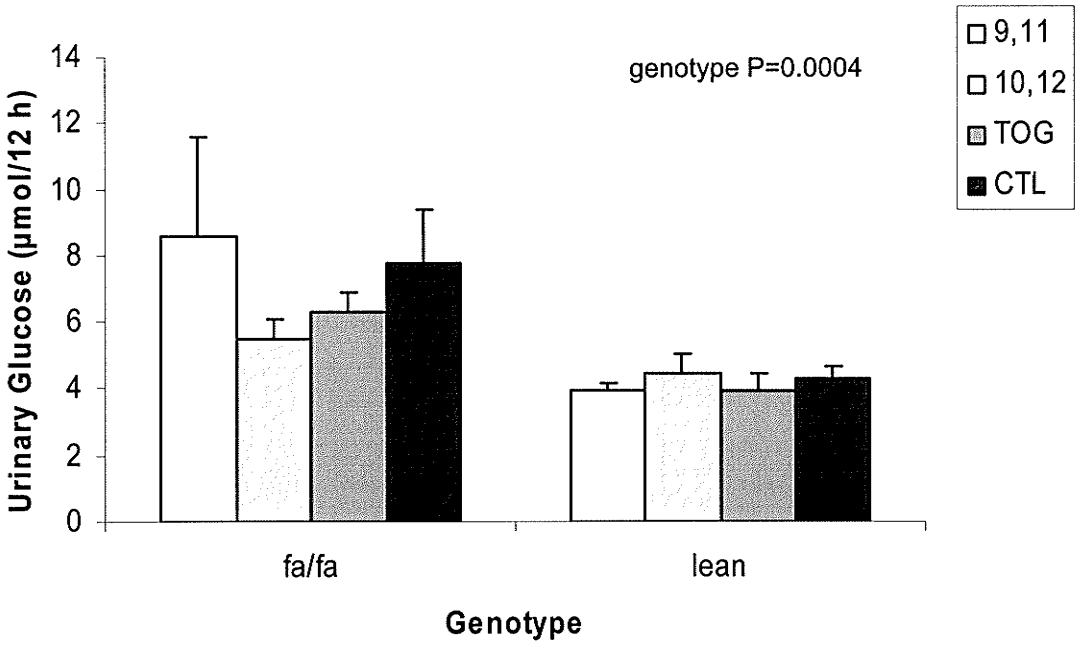


Figure 13 Urine glucose excreted in 12 hours for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10 except for *fa/fa* 9,11 where n=9). The four different diets and statistical analysis are as described in Figure 5.

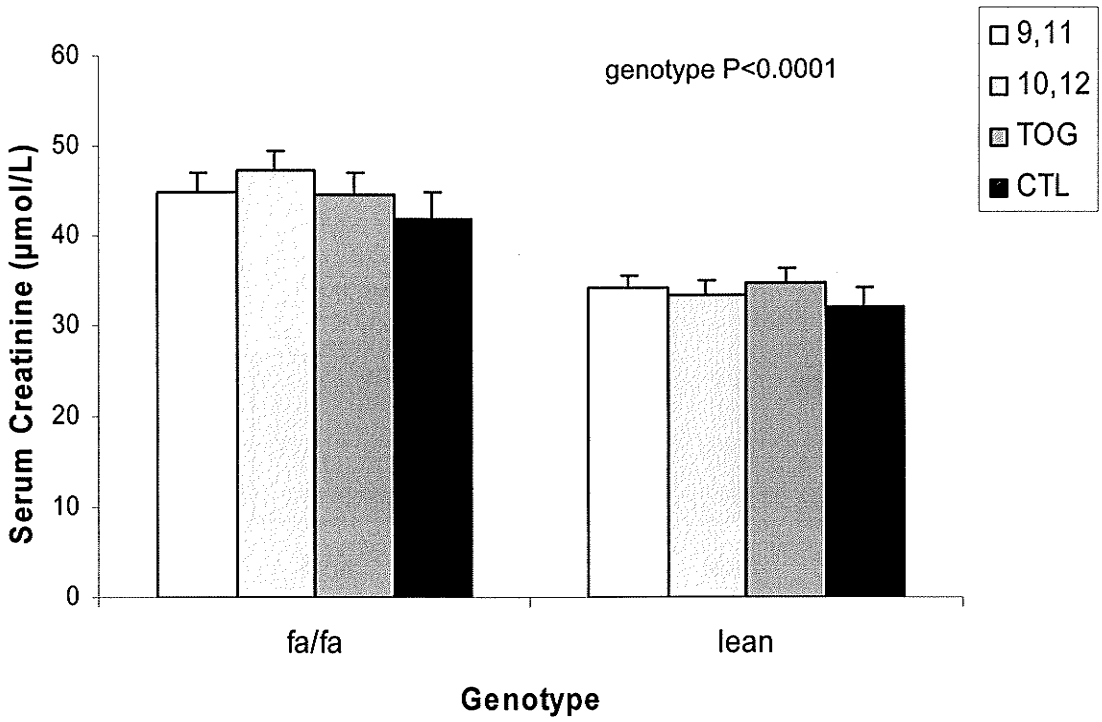


Figure 14 Serum creatinine for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets and statistical analysis are as described in Figure 5.

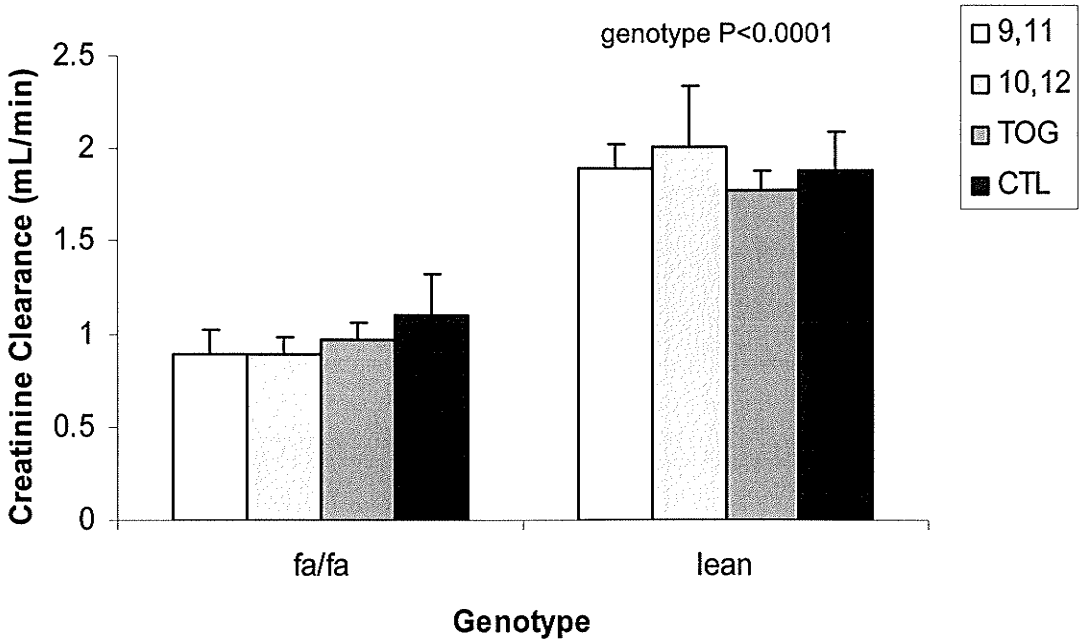


Figure 15 Creatinine clearance for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10 except for lean TOG where n=9). The four different diets and statistical analysis are as described in Figure 5.

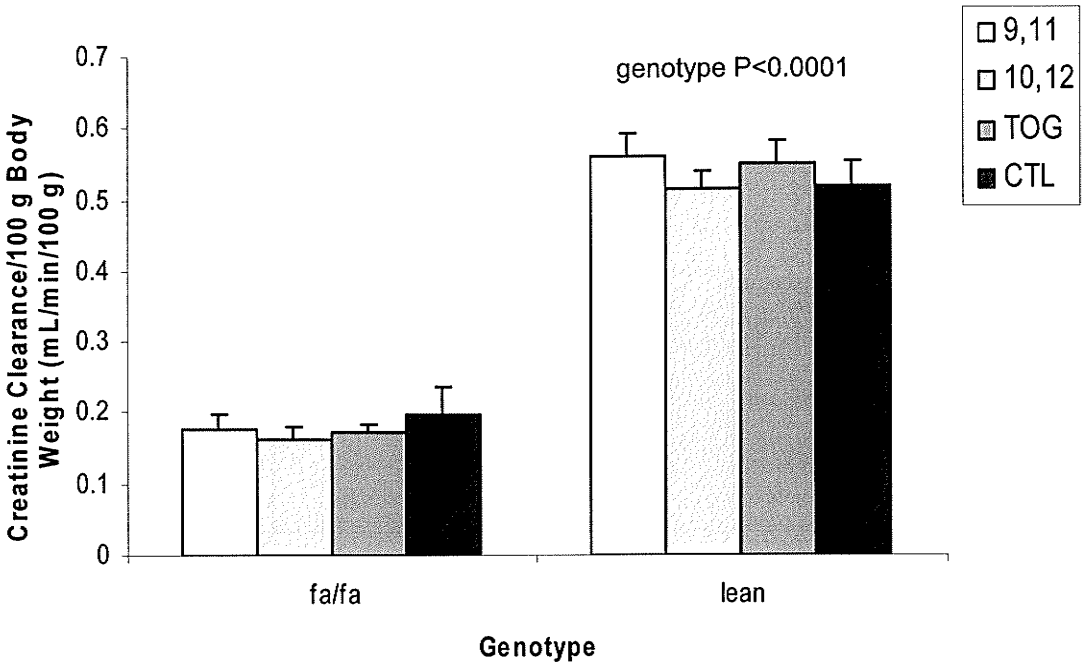


Figure 16 Creatinine clearance expressed per 100 g body weight for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10 except for lean 10,12; TOG and CTL and *fa/fa* 9,11 where n=9). The four different diets and statistical analysis are as described in Figure 5.

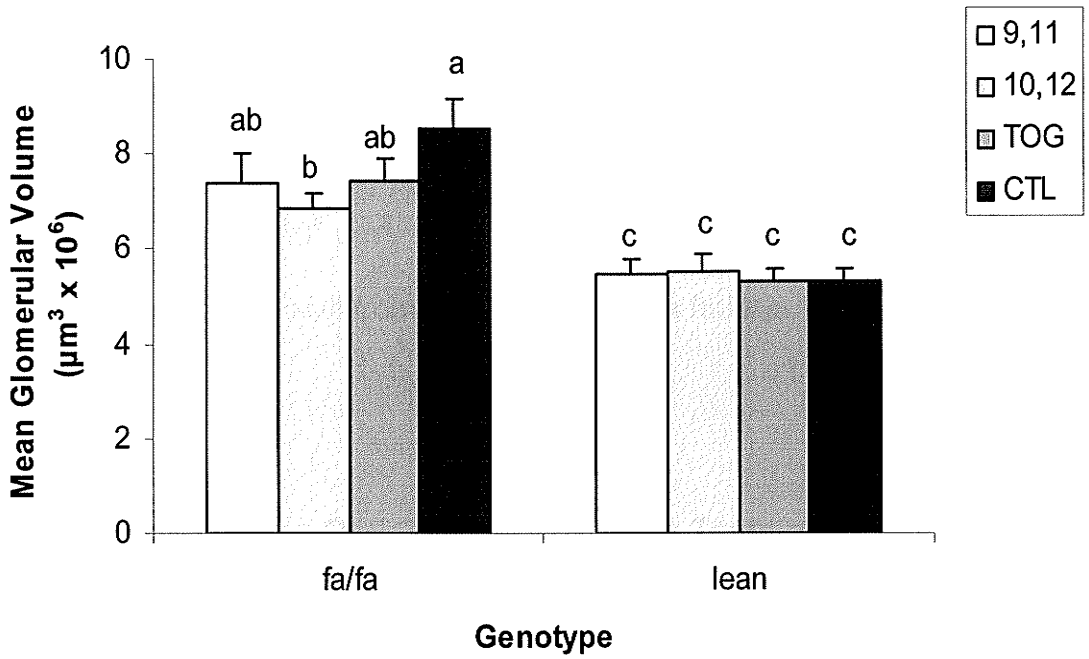


Figure 17 Mean glomerular volume (MGV) from kidney cross sections for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets and statistical analysis are as described in Figure 5. Significant CONTRAST lean versus *fa/fa* for 10,12 versus CTL, P=0.0624.

fa/fa rats also had a larger MGV compared to lean rats for all diets, indicating the presence of glomerular hypertrophy. For example, the *fa/fa* rats on the CTL diet had a MGV of 8.54 $\pm$ 0.64 x 10^6 μm^3, while the lean rats on the CTL diet had a MGV of 5.34 $\pm$ 0.27 x 10^6 μm^3. Please refer to **Figure 18** for representative cross sections of glomeruli from *fa/fa* and lean rats fed the 10,12 and the CTL diets.

Serum Glucose During Oral Glucose Tolerance Testing (OGTT)

Area under the curve for serum glucose during the OGTT was calculated and there was a significant interaction between diet x genotype (P=0.0238) from the ANOVA. Oral glucose tolerance was improved in *fa/fa* rats fed the TOG or the 10,12 diet compared to control, but not in those fed the 9,11 isomer alone (**Figure 19**). The pre-planned contrasts for lean versus *fa/fa* for 10,12 versus CTL (P=0.0531), lean versus *fa/fa* for TOG versus CTL (P=0.0039) and lean versus *fa/fa* for 9,11 versus TOG (P=0.0413) were all in agreement with this. The *fa/fa* rats had impaired oral glucose tolerance compared to lean rats for all diets. For example, the *fa/fa* rats on the CTL diet had an area under the curve for serum glucose of 1059 $\pm$ 48 $mmol*L^{-1}*min$, while the lean rats on the CTL diet had an area under the curve for serum glucose of 622 $\pm$ 25 $mmol*L^{-1}*min$.

Repeated measures indicated that there was an interaction of time x genotype x diet (P<0.0001) for serum glucose over time. At t=0, *fa/fa* rats fed the 10,12 diet had a higher serum glucose concentration compared to all other groups. At t=15, serum glucose concentrations were lower in *fa/fa* rats fed the

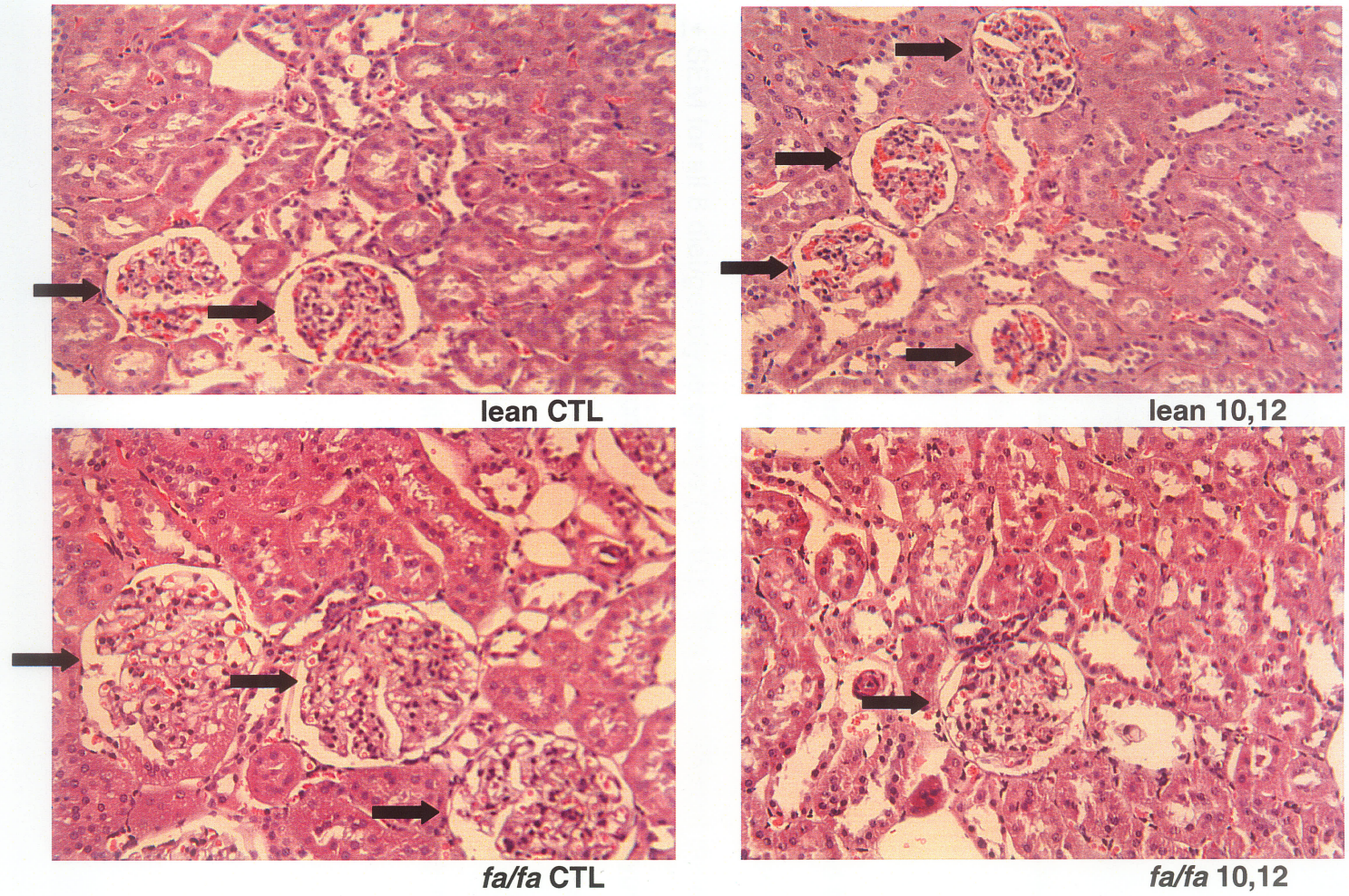


Figure 18: Longitudinal cross sections of kidney glomeruli stained with hematoxylin and eosin. Pictures show glomeruli of rats fed the 10,12 and CTL diets at 20X magnification. Diets are described in Figure 5. Refer to Figure 17 for the statistics (CONTRAST lean versus *fa/fa* for 10,12 versus CTL, P=0.0624).

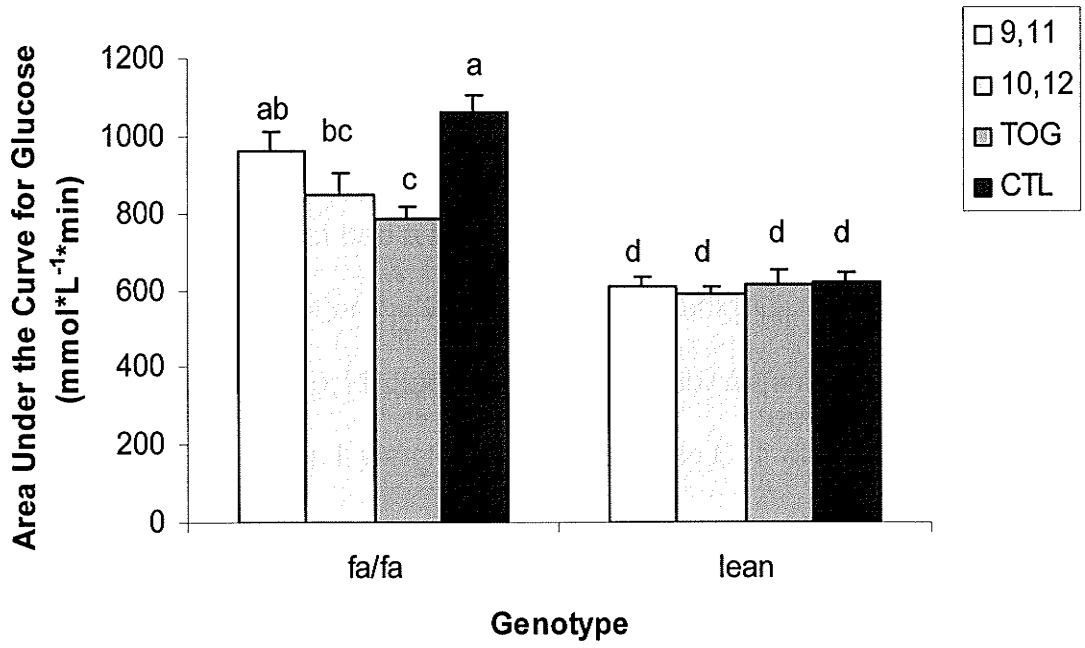


Figure 19 Area under the curve for serum glucose of *fa/fa* and lean Zucker rats during an oral glucose tolerance test (OGTT). Bars represent the mean ± SEM for all 8 diet/genotype groups (n=10). The four different diets and statistical analysis are as described in Figure 5. Significant genotype x diet interaction, P=0.0238 and significant CONTRAST lean versus *fa/fa* for 10,12 versus CTL, P=0.0531, significant CONTRAST lean versus *fa/fa* for TOG versus CTL, P=0.0039, and significant CONTRAST lean versus *fa/fa* for 9,11 versus TOG, P=0.0413.

9,11 and TOG diets compared to *fa/fa* rats fed the CTL diet. At t=30, serum glucose concentrations were lower in *fa/fa* rats fed the 10,12 or the TOG diet compared to *fa/fa* rats fed the CTL or the 9,11 diet. At t=60, *fa/fa* rats fed the 10,12 diet or the TOG diet had a lower serum glucose concentration compared to *fa/fa* rats fed the CTL diet or the 9,11 diet. The serum glucose concentration at t=60 for *fa/fa* rats on the 9,11 diet was higher than for *fa/fa* rats fed the 10,12 or TOG diets, but lower than it was for *fa/fa* rats fed the CTL diet (**Figure 20**). The *fa/fa* rats, overall, had significantly higher serum glucose concentrations compared to lean rats for all diets.

Protein Expression of Enzymes Involved in Eicosanoid Production

A genotype effect existed for protein expression of cytosolic $cPLA_2$ in that the *fa/fa* rats had a higher protein expression of cytosolic $cPLA_2$ compared to lean rats (0.31 ± 0.04 arbitrary units versus 0.23 ± 0.03 arbitrary units, respectively, P=0.0232, **Figure 21**). **Figure 26** shows representative protein bands from western immunoblotting.

There were no significant effects for particulate $cPLA_2$ (**Figure 22**). **Figure 26** shows representative protein bands from western immunoblotting.

The ratio of cytosolic $cPLA_2$ to particulate $cPLA_2$ was then calculated and it was found that the *fa/fa* rats had a higher cytosolic $cPLA_2$/particulate $cPLA_2$ ratio compared to the lean rats (0.52 ± 0.10 arbitrary units versus 0.27 ± 0.05 arbitrary units, respectively, P=0.0090, **Figure 23**).

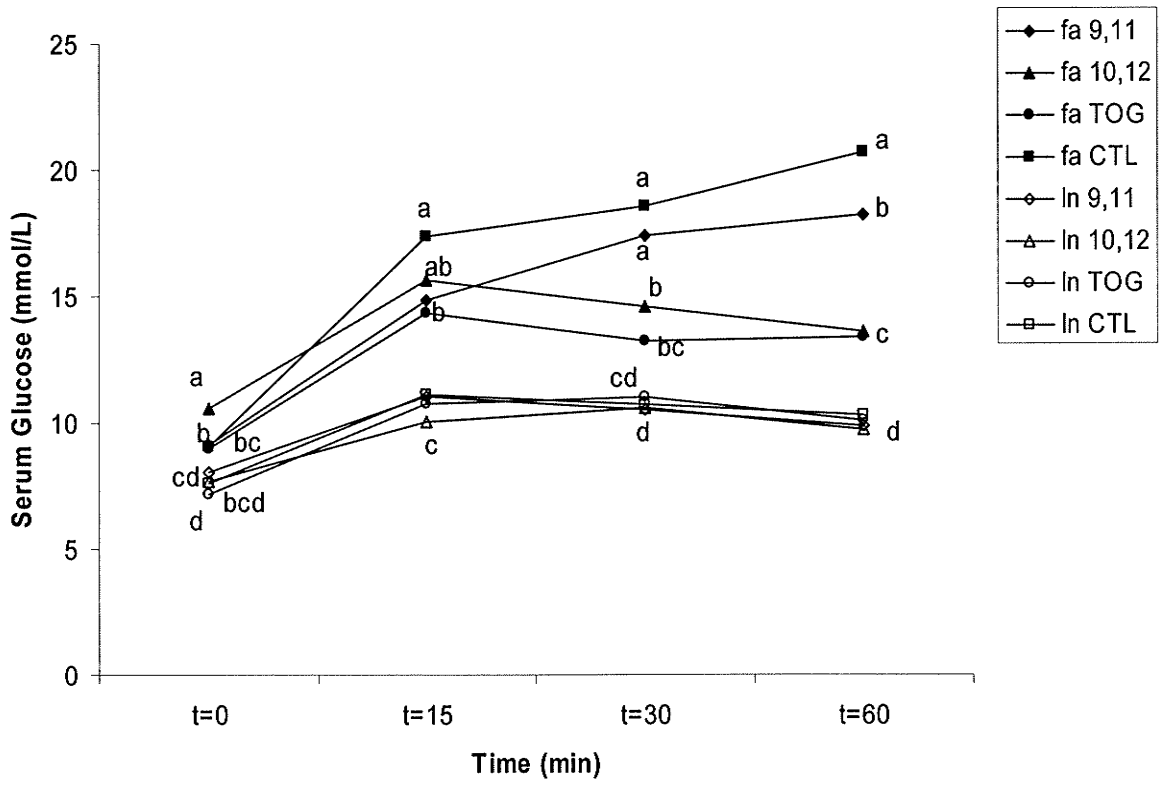


Figure 20 Serum glucose concentration at various time points before and after an oral glucose load of 1 g/Kg body weight in *fa/fa* and lean Zucker rats. Symbols represent the mean for all 8 diet/genotype groups (n=10) at each time point. The four different diets are as described in Figure 5. Data were analyzed as repeated measures. Different letters indicate means are significantly different as determined by Duncan's Multiple Range Test. Significant time x genotype x diet interaction, $P<0.0001$. In cases where means are close together or overlapping, only one Duncan's letter is used to represent that the means are not significantly different from one another. Duncan's letters are as follows:

[t=0; fa 9,11 (b), fa 10,12 (a), fa TOG (bc), fa CTL (bc), ln 9,11 (bcd), ln 10,12 (bcd), ln TOG (d), ln CTL (cd); t=15; fa 9,11 (b), fa 10,12 (ab), fa TOG (b), fa CTL (a), all lean animals (c); t=30; fa 9,11 (a), fa 10,12 (b), fa TOG (bc), fa CTL (a), ln 9,11 (d), ln 10,12 (d), ln TOG (cd), ln CTL (d); t=60; fa 9,11 (b), fa 10,12 (c), fa TOG (c), fa CTL (a), all lean animals (d)].

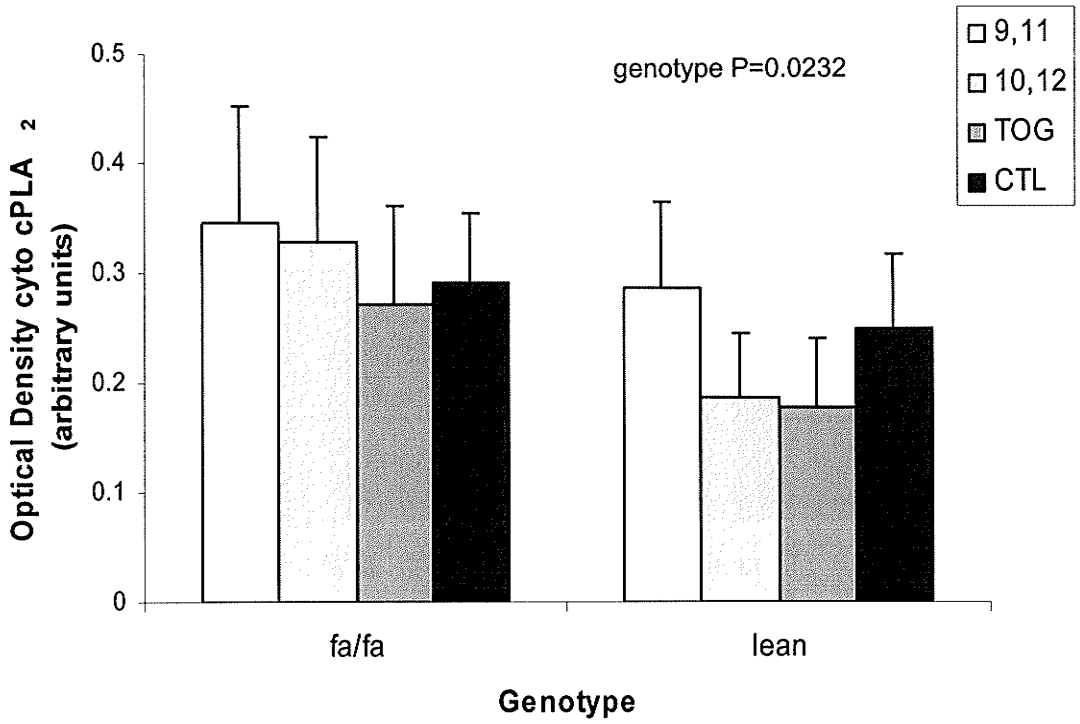


Figure 21 Protein expression for cytosolic $cPLA_2$ (cyto $cPLA_2$) for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=9 except for *fa/fa* 9,11 where n=8). The four different diets and statistical analysis are as described in Figure 5.

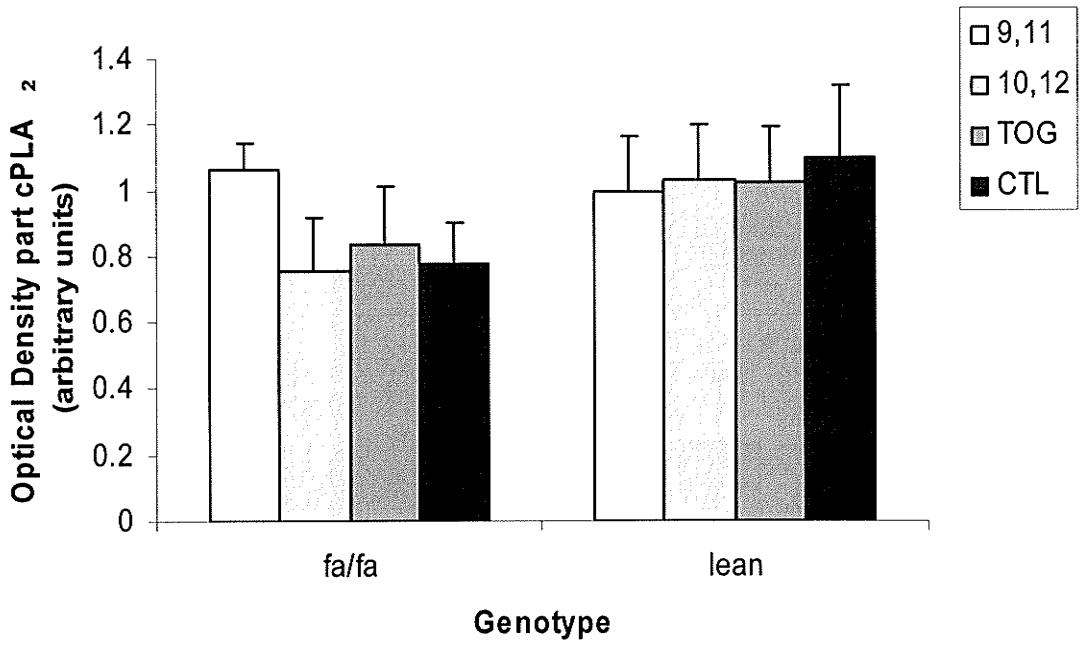


Figure 22 Protein expression for particulate $cPLA_2$ (part $cPLA_2$) for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=9). The four different diets and statistical analysis are as described in Figure 5. There were no significant effects.

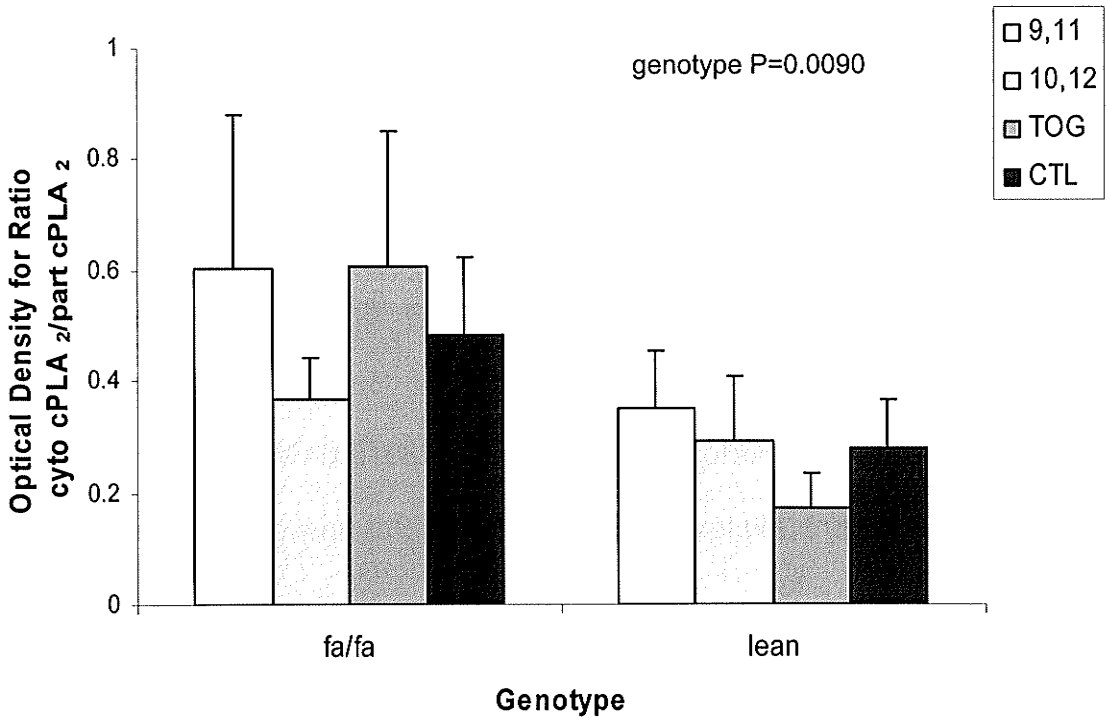


Figure 23 Protein expression of the ratio of cytosolic $cPLA_2$ to particulate $cPLA_2$ (cyto $cPLA_2$/part $cPLA_2$) for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=9 except *fa/fa* 10,12 where n=8). The four different diets and statistical analysis are as described in Figure 5.

As shown by **Figure 24**, there were no significant effects for COX-1. **Figure 26** shows representative protein bands from western immunoblotting.

However, as depicted in **Figure 25**, a genotype x diet interaction existed (P=0.0991) from the ANOVA for COX-2 showing *the fa/fa* rats on the 10,12 diet having a lower COX-2 protein expression compared to *fa/fa* rats on the CTL diet. The pre-planned contrast lean versus *fa/fa* for 10,12 versus CTL was also in agreement with this, (P=0.0198). COX-2 protein expression was also elevated in *fa/fa* rats compared to lean rats for all diets. For example, the protein expression for COX-2 for *fa/fa* rats given the CTL diet was 1.89 $\pm$ 0.32 arbitrary units versus a protein expression of 0.29 $\pm$ 0.05 arbitrary units for lean rats given the CTL diet. **Figure 26** shows representative protein bands from western immunoblotting.

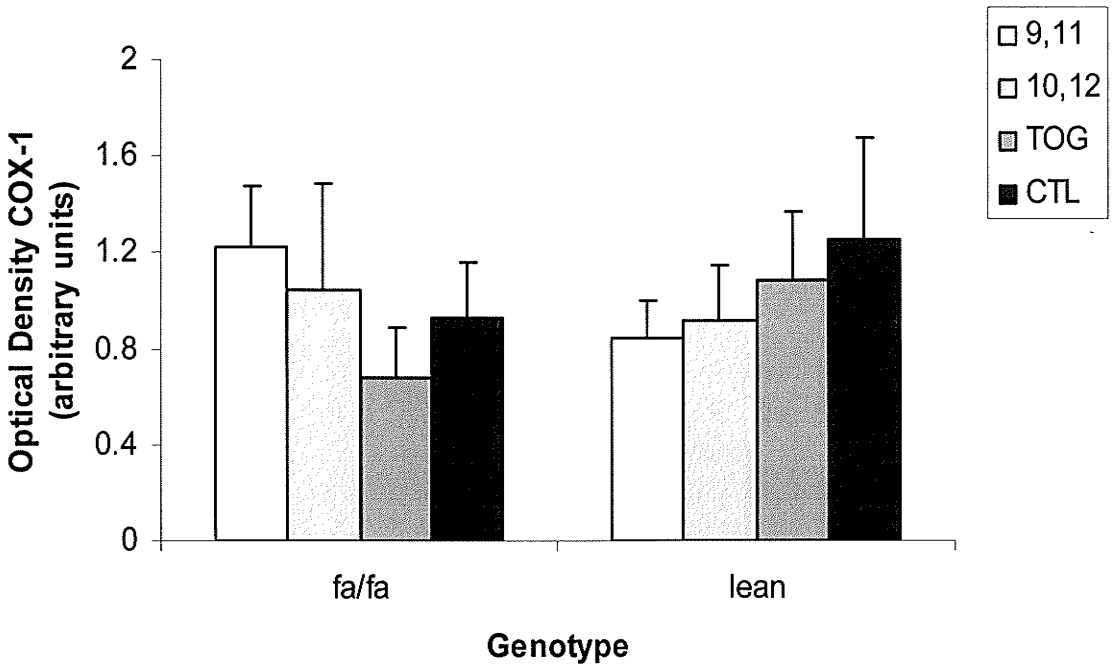


Figure 24 Protein expression for COX-1 for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=9 except for *fa/fa* 10,12 and lean 10,12 where n=8, lean TOG and lean CTL where n=7 and lean 9,11 where n=6). The four different diets and statistical analysis are as described in Figure 5. There were no significant effects.

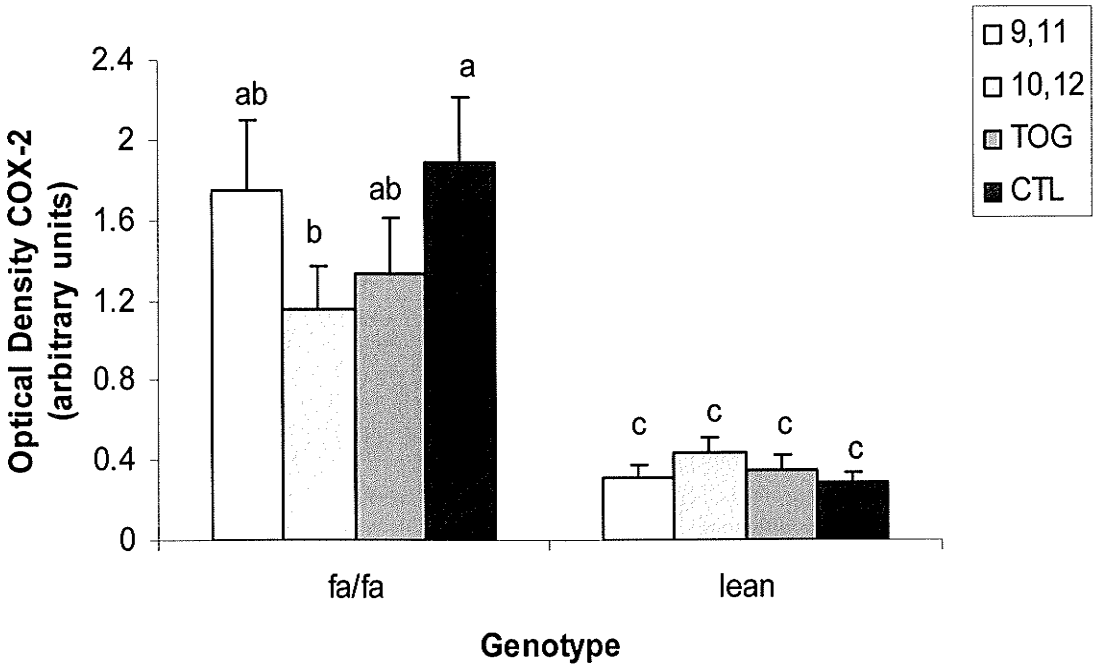


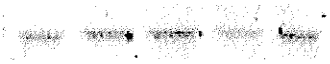
Figure 25 Protein expression for COX-2 for *fa/fa* and lean Zucker rats. Bars represent the mean ± SEM for all 8 diet/genotype groups (n=9). The four different diets and statistical analysis are as described in Figure 5. Significant genotype x diet interaction, P=0.0991 and significant CONTRAST lean versus *fa/fa* for 10,12 versus CTL, P=0.0198.

***fa/fa* rats**

cytosolic $cPLA_2$ [1]

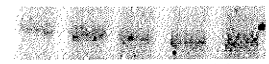
std* 10,12 TOG 9,11 CTL

particulate $cPLA_2$



10,12 TOG 9,11 CTL std

COX-1 [2]



TOG CTL 10,12 9,11 std

COX-2 [3]

CTL 9,11 TOG 10,12 std

lean rats

cytosolic $cPLA_2$

CTL 9,11 TOG 10,12 std

particulate $cPLA_2$

TOG CTL 10,12 9,11 std

COX-1

TOG 9,11 CTL 10,12 std

COX-2

std CTL 9,11 TOG 10,12

Figure 26 Representative protein bands from western immunoblotting analysis for cytosolic $cPLA_2$, particulate $cPLA_2$, COX-1 and COX-1. Diets are as described in Figure 5.

[1] $cPLA_2$ = cytosolic phospholipase A_2
[2] COX-1 = cyclooxygenase-1
[3] COX-2 = cyclooxygenase-2
* std = standard

DISCUSSION

Feed Intake and Body Weight

The *fa/fa* rats on the 10,12 diet had a lower total feed intake compared to the *fa/fa* rats on all other diets (**Figure 5**). Also, the *fa/fa* rats on the TOG diet ate significantly less than *fa/fa* rats on the CTL diet (**Figure 5**). This is interesting and suggests that the *t*10, *c*12 isomer may lead to greater satiety and therefore a reduced feed intake in this rat model. Implications of this finding are that perhaps this isomer may be useful in aiding with portion control for people who are trying to eat less. Recently, a double-blind, placebo-controlled study in overweight men and women found that supplementation with a CLA mixture (1.8 or 3.6 g/day) for 13 weeks after completing a 3 week very low calorie diet resulted in greater satiety and feelings of fullness compared to supplementation with oleic acid, which served as a control (Kamphuis et al., 2003). This occurred independently of the dose of CLA. However, energy intake at breakfast, measured at week 13, did not differ between CLA and control groups, and CLA supplementation did not improve weight maintenance after the initial weight loss (Kamphuis et al., 2003). Since a CLA mixture was used in this study, it is not possible to single out which isomer was responsible for elevating feelings of satiety in these overweight subjects.

The reduced feed intake of the *fa/fa* rats on the TOG diet compared to *fa/fa* rats on the CTL diet in this study (**Figure 5**) is similar to results observed by Ryder et al. (2001) in the ZDF rat model. These authors demonstrated that ZDF rats fed a 50:50 CLA mixture diet containing predominantly the *c*9, *t*11 and *t*10,

*c*12 isomers had a significantly lower feed intake compared to rats fed the control diet and rats fed the *c*9, *t*11 isomer alone. The lower feed intake in the 50:50 isomer group began on day 4 of feeding and continued until the end of the study (day 14). Although the CLA isomers were not fed separately in this study, it seems likely that the *t*10, *c*12 CLA isomer was the one responsible for reducing feed intake in the ZDF rat model as a reduced feed intake was not observed in the rats fed the *c*9, *t*11 isomer alone (Ryder et al., 2001).

In contrast to these results, the study by Warford (2003) found that supplementation with a 1.5% CLA mixture fed for 8 weeks to Zucker rats had no overall effect on total feed intake. Perhaps this is because the mixture used by Warford (2003) was not simply a 50:50 mixture. In fact, the mixture contained approximately 29% of the *c*9, *t*11 isomer, 30% of the *t*10, *c*12 isomer, 18% of the *c*11, *t*13 isomer and 16% of the *t*8, *c*10 isomer and was approximately 86% pure (Warford, 2003). The latter 2 isomers were present in a large enough quantity that they may be able to explain the discrepancy observed regarding the effect of CLA on total feed intake between this study and the current study.

In the present study, the *fa/fa* rats had a higher total feed intake compared to the lean rats for all diets (**Figure 5**). Since the *fa/fa* Zucker rat is hyperphagic (Bray, 1977), a greater total feed intake is to be expected. The hyperphagia observed in this model is a result of a mutation in the leptin receptor (Zucker & Zucker, 1961). Since leptin (a hormone responsible for maintaining a balance between energy expenditure and food intake) does not function properly in this model, *fa/fa* Zucker rats essentially cannot control excessive eating and thus

rapidly gain weight and become obese. The study by Warford (2003) indicated that *fa/fa* Zucker rats ate a total of 1539 ± 27 g of feed, and the lean Zucker rats ate a total of 1078 ± 22 g of feed, during the 8 week study period. This is in accordance with the present study in which the *fa/fa* rats on the CTL diet ate a total of 1666 ± 51 g of feed and the lean rats on the CTL diet ate a total of 1022 ± 24 g of feed over the 8 week study period.

The fact that the *fa/fa* rats on the 10,12 diet had a lower feed intake compared to *fa/fa* rats on all other diets (**Figure 5**) would lead us to believe that perhaps their final body weights would be lower as well. In fact, this was not the case, as final body weight among the *fa/fa* rats was not significantly altered by diet (**Figure 6**). This does not agree with the results from the study by Ryder et al. (2001) where ZDF rats in the 50:50 CLA isomer group had a lower final body weight compared to ZDF rats in the *c*9, *t*11 CLA isomer group or the control group. This poses the question as to why a reduced feed intake did not lead to a subsequent reduced body weight in the *fa/fa* Zucker rats fed the 10,12 diet. A possible explanation is that the *t*10, *c*12 isomer resulted in a greater feed efficiency in this model, although this is only speculation, as there are not any studies in this model to support this. In fact, Akahoshi et al. (2002) found that feeding a 1% CLA mixture (containing mostly the *c*9, *t*11 and *t*10, *c*12 isomers) to male 3 week old Crj:CD-1 mice for 8 weeks resulted in a reduced feed efficiency compared to mice fed linoleic acid. Conclusions about feed efficiency in this mouse study, however, cannot be extrapolated to the *fa/fa* Zucker rat model

because as mentioned in the literature review, effects of CLA on feed intake and body composition vary depending on the animal model used.

At the end of the 8 week study, the *fa/fa* rats weighed 67% more than the lean rats. The *fa/fa* rats weighed more than the lean rats at 14 weeks of age because they ate more feed, without increasing energy expenditure. These results are comparable with those from Warford (2003) where at the end of the 8 week study, the *fa/fa* rats weighed 58% more than lean rats.

Kidney Weight

It is interesting to note that in the current study, the *fa/fa* rats on the 10,12 diet had a lower kidney weight than *fa/fa* rats on the CTL diet [pre-planned contrast (P=0.067), **Figure 7**]. This is difficult to explain, because as noted above, body weights were not significantly different among the *fa/fa* rats. The *fa/fa* rats on the CTL diet had larger kidneys than *fa/fa* rats on the 10,12 diet and also had a larger MGVs compared to *fa/fa* rats on the 10,12 diet. A possible explanation is that having enlarged glomeruli could result in larger kidneys in *fa/fa* rats given the CTL diet. Perhaps the *t*10, *c*12 group has kidneys that are more characteristic of "normal" kidneys compared to *fa/fa* CTL rats, as kidney weight in the 10,12 group more closely resembled the kidney weight of lean controls. As reviewed by Parving et al. (1996), an increase in renal size (or nephromegaly) early in DN, has been documented in patients with DM-1. This, however, has not been documented in patients with DM-2, at least not in those who have had the disease for only a short while (Parving et al., 1996).

This study supports, as well as adds new insight to what was reported by Warford in 2003. This author was also able to demonstrate that *fa/fa* rats fed a CLA mixture had a significantly lower kidney weight compared to *fa/fa* rats fed the control diet. The current study furthers this finding as it shows that the *t*10, *c*12 CLA isomer is responsible for this effect.

At 14 weeks of age, the *fa/fa* rats had a larger kidney weight compared to lean rats for all diets (**Figure 7**). This is consistent with what has been reported in the literature. Kasiske et al. (1985) found that at 14 weeks of age, *fa/fa* Zucker rats had a 24% larger kidney weight compared to age-matched lean control Zucker rats. The actual values for *fa/fa* and lean kidney weights in the Kasiske study were 2.40 $\pm$ 0.24 g and 1.93 $\pm$ 0.18 g for *fa/fa* and lean 14 week old Zucker rats, respectively. These values are lower than the values obtained from the current study where 14 week old *fa/fa* and lean Zucker rats fed the CTL diet had kidney weights of 3.0 $\pm$ 0.1 g and 2.3 $\pm$ 0.1 g, respectively. This may be explained by the fact that the *fa/fa* and lean rats in the Kasiske study had lower body weights at 14 weeks of age (480 $\pm$ 57 g and 311 $\pm$ 48 g, respectively) compared to the *fa/fa* and lean rats in the current study (554 $\pm$ 7 g and 331 $\pm$ 3 g, respectively). Perhaps the rats in the Kasiske study had not grown as much as the rats in the current study by 14 weeks of age.

When kidney weight was expressed per 100 g body weight, the significance of the 10,12 diet disappeared, in that the *fa/fa* rats on the 10,12 diet no longer had a different kidney weight per 100 g body weight compared to any other *fa/fa* group (**Figure 8**). A different pre-planned contrast was, however,

significant in that the difference between lean and *fa/fa* rats for the TOG diet was different from the difference between lean and *fa/fa* rats for the CTL diet, although the Duncan's Multiple Range Test was not in agreement with this.

When kidney weight was expressed relative to 100 g body weight, a genotype effect existed, in that the *fa/fa* rats had a 38% lower kidney weight per 100 g body weight compared to lean rats for all diets. This is somewhat hard to interpret because the *fa/fa* Zucker rat is an obese model, and therefore displays a significantly higher amount of body fat compared to lean Zucker rats (Bray, 1977). This renders the comparison difficult as the lean kidneys are being compared to mostly lean body mass whereas the *fa/fa* kidneys are being compared to mostly fat mass. A study by Mathey et al. (2002) indicates that the percentage of lean mass in 6 month old male *fa/fa* Zucker rats is significantly less than the percentage of lean mass in their non-obese counterparts (35.7 $\pm$ 1.3% versus 71.3 $\pm$ 0.9%, respectively), as measured by dual energy X-ray absorptiometry. If the body composition of the rats in the current study was similar to that observed in the study by Mathey et al. (2002), the kidney weight to lean body mass ratio would be substantially larger in the *fa/fa* Zucker rats as compared to the lean Zucker rats.

Urinary Parameters

The amount of urine excreted in 12 hours was 47% higher in *fa/fa* compared to lean rats (**Figure 9**). This is slightly higher but still in the range with what was reported in the study by Warford (2003) where the 12-hour urinary

excretion was 32% higher in *fa/fa* compared with lean Zucker rats. A higher amount of urine excretion in the *fa/fa* Zucker rats is expected as it has been reported in the review by Bray (1977) that the kidneys of *fa/fa* Zucker rats have a decreased concentrating capacity. Glycosuria was observed in the *fa/fa* Zucker rat in the current study and has also been reported in this model in the literature (Aprikian et al., 2002). This could also explain the higher urine volume observed in this model as water would follow glucose into the urine due to osmosis.

Total urinary protein excretion in 12 hours was 76% higher in *fa/fa* compared to lean rats (**Figure 10**). This is not surprising as a high urinary protein is a characteristic of this model as reviewed by Bray (1977). However, in the study by Coimbra et al. (2000), proteinuria was not evident in 14 week old *fa/fa* Zucker rats compared to lean controls. It was, however, markedly increased in 40 week old *fa/fa* rats compared to age-matched lean controls, and may have been increased earlier, but there was no way of knowing this as age-matched lean controls were only available at 6, 14 and 40 weeks of age (Coimbra et al., 2000). It can be argued that in the present study, the *fa/fa* rats had a higher total urinary protein excretion because they had a higher total volume of urine excreted. It is therefore important to express urine protein relative to urine creatinine in order to remove the urine volume factor. Total urine creatinine will first be discussed, followed by urine protein expressed relative to urine creatinine.

Total urinary creatinine excreted in 12 hours was 31% lower in *fa/fa* rats compared to lean rats (**Figure 11**). This makes sense because the *fa/fa* rats are

known to have altered renal structure (as evidenced by larger glomerular area) and impaired renal function (as evidenced by albuminuria) as early as 14 weeks of age, compared to their lean littermates (Kasiske et al., 1985). If the kidneys of the *fa/fa* rats are not functioning properly, a lower urine creatinine excretion would be expected and would imply that the creatinine may be accumulating in the blood. The results from the present study are in excellent agreement with the study by Warford (2003), where the *fa/fa* rats also had a 31% lower 12-hour urinary creatinine excretion compared to lean rats.

When urinary protein was expressed relative to urinary creatinine, a genotype effect existed in that the *fa/fa* rats had a 164% higher urinary protein/creatinine ratio compared to the lean rats (**Figure 12**). The study by Warford (2003) found that the *fa/fa* rats had a 109% higher protein/creatinine ratio compared to lean rats.

There was also a significant diet effect in that the urinary protein/creatinine ratio was significantly elevated in rats fed the 9,11 and TOG diets relative to the CTL diet, but was not significantly elevated in rats fed the 10,12 diet (**Figure 12**). This implies that the *t*10, *c*12 CLA isomer may be exerting a protective effect in the kidneys of both lean and obese Zucker rats by preventing an elevation in urinary protein relative to urinary creatinine. This is beneficial in relation to kidney disease, as it is known that protein in the urine is one of the earliest manifestations of DN (Loon, 2003), and is also a sign of impaired renal function in patients with the metabolic syndrome (Chen et al., 2004).

It is not known why the *c*9, *t*11 isomer given alone and the TOG diet were causing an elevation in urine protein/creatinine compared to the CTL diet. This is harmful to the kidney and could lead to a faster progression to renal failure. In contrast to this, Park et al. (2005) found that a 1% (w/w) CLA mixture given for 18 months to weanling male Fisher 344 rats reduced the amount of protein in the urine measured at 70 weeks of feeding. This may be important as all animals had renal failure at the end of the study. However, this was not statistically significant (P=0.06). CLA also significantly reduced fasting and fed blood glucose in these animals (Park et al., 2005). In the current study, the elevation in protein/creatinine seems to be related to the *c*9, *t*11 isomer as an elevation in protein/creatinine ratio was not seen in rats fed the 10,12 diet.

A genotype effect was present in the current study for urinary glucose. The *fa/fa* rats had a 68% higher 12-hour urinary glucose excretion compared to lean rats (**Figure 13**). Measurement of urinary glucose is seen as a cost-effective screening tool for diabetes, and since 1994, has been performed annually on all elementary, middle school and high school students in Japan as a result of the increasing prevalence of DM-2 among youth (Yokota et al., 2004). According to these authors, the cost is only about $2 U.S. per student. This cost also covers urinary screening for kidney diseases (which has been ongoing since 1974 in elementary and junior high schools), so in fact screening for urinary glucose is only a portion of this cost (Yokota et al., 2004). Although the *fa/fa* Zucker rat is only hyperinsulinemic (Bray, 1977), but not hyperglycemic so does not have overt DM-2, urine glucose excretion may still predict progression of

kidney disease. For example, a study by Pijls et al. (2001) found that in patients with DM-2, differences in glycosuria as well as other factors independently explained a significant portion of the variation in ACR that existed between subjects. More specifically, patients with albuminuria had a significantly higher urinary glucose concentration compared to those with normal albumin excretion (Pijls et al., 2001). Although CLA did not have a significant effect on urinary glucose in the present study, the *fa/fa* rats fed the 10,12 diet tended to have a urinary glucose excretion that approached that of the lean rats, perhaps suggesting improved metabolic control in these animals.

Serum Creatinine and Creatinine Clearance

The *fa/fa* rats had a 33% higher serum creatinine concentration compared to lean rats (**Figure 14**). Based on the urinary creatinine results (lower in *fa/fa* compared to lean rats), this data appears logical. Since creatinine cannot be properly excreted in the urine due to impaired renal function in the *fa/fa* rats, it is building up in the serum of the *fa/fa* rats to a greater extent than in lean rats. This is in agreement with the study by Warford (2003), where the *fa/fa* rats had a 55% higher serum creatinine concentration compared to lean rats. Urine or serum creatinine alone is not very informative, therefore, creatinine clearance, as an estimation of GFR (National Kidney Foundation, 2002) was calculated.

A genotype effect existed for creatinine clearance in that it was 49% lower in *fa/fa* rats compared to lean rats (**Figure 15**). Creatinine clearance (as determined by a prediction equation) is used clinically in order to estimate the

GFR, or more specifically, it is used to assess kidney function (National Kidney Foundation, 2002). It must be stressed that creatinine clearance is an estimation of GFR because the gold standard for measuring GFR is inulin clearance. This is difficult to measure clinically and it is expensive as it requires intravenous infusion along with collections of timed urine samples lasting several hours (National Kidney Foundation, 2002). Although inulin clearance may give a more reliable measure of GFR, it was not feasible in the present study. Warford (2003) also found that creatinine clearance (as determined by the same formula) was 66% lower in *fa/fa* rats compared to lean rats.

When creatinine clearance was adjusted to 100 g body weight, the *fa/fa* rats still displayed a lower creatinine clearance compared to the lean rats (**Figure 16**). Creatinine clearance was 67% lower in *fa/fa* compared to lean rats. This adjustment was carried out in order to account for body size of the animal. It is known that body size, and more specifically a larger muscle mass, can increase serum creatinine (National Kidney Foundation, 2002). Since lean muscle mass data was not available for the current study, adjustment for this confounding factor cannot be carried out.

Mean Glomerular Volume (MGV)

The *fa/fa* rats on the 10,12 diet had a 20% smaller MGV than the *fa/fa* rats on the CTL diet [pre-planned contrast ($P=0.0624$), **Figures 17 and 18**]. The Duncan's Multiple Range Test was in agreement with this ($P<0.05$). Since a higher MGV is indicative of impaired renal function in the *fa/fa* Zucker rat

(Kasiske et al., 1985; Maddox et al., 2002), attenuating this enlargement by the *t*10, *c*12 CLA isomer in this rat model is beneficial. Warford et al (2003) also demonstrated that in the *fa/fa* Zucker rat, feeding a 1.5% (w/w) CLA mixture for 8 weeks resulted in a 28% smaller MGV compared to feeding the control diet. The CLA fed in the study by Warford (2003) was a 1.5% (w/w) mixture, containing four isomers. This may be a reason why a larger reduction was seen in that study compared to the present study, which used single CLA isomers and a 2-isomer mixture at a level of 0.4%, and 0.8%, respectively. The current study brings new insight as to which CLA isomer is responsible for the beneficial effect on MGV.

Glomerular hypertrophy was present in the *fa/fa* Zucker rats as evidenced by a 39% larger MGV compared to lean rats for all diets (**Figure 17**). This is in agreement with the study by Kasiske et al. (1985). These researchers reported that *fa/fa* rats had a larger glomerular area at 14 weeks of age compared to age-matched lean control rats. The glomerular area followed the trend of increasing over time (14, 28 and 68 weeks) in both *fa/fa* and lean rats, although the glomerulus tended to always be larger in the obese animals (Kasiske et al., 1985). Maddox et al. (2002) determined the MGV in 60 week old female *fa/fa* and lean Zucker rats and found that the MGV was 90% greater in ad libitum fed *fa/fa* rats compared to age-matched lean controls. The higher percent difference of this study compared to the current study may be due to the increased age of the animals (60 weeks old versus 14 weeks old, respectively), with the older animals having more pronounced renal damage. Dietary feed restriction by

these researchers in 6 week old and 12 week old obese rats to the intakes of lean rats (14 g/day) significantly reduced the MGV measurements so that they resembled those of lean rats. The improvements in MGV measurements were associated with a reduced body weight as final body weight was about 30% lower in obese rats that were feed restricted at 6 or 12 weeks of age compared to obese rats fed ad libitum (Maddox et al., 2002). This suggests that obesity, and its associated metabolic abnormalities contribute to renal damage as evidenced by glomerular hypertrophy.

In summary, renal impairment was present in the *fa/fa* rats compared to the lean rats as evidenced by the above-mentioned parameters. These measurements were consistent in demonstrating renal injury in the *fa/fa* Zucker rat model. Relative to lean rats, the *fa/fa* rats had a greater kidney weight, a higher 12-hour urine excretion, an elevated 12-hour urine protein excretion, a lower 12-hour urine creatinine excretion and a higher 12-hour urine protein/creatinine ratio. They also showed a greater 12-hour urine glucose excretion, a higher serum creatinine and a reduced creatinine clearance, and kidney glomeruli showed evidence of hypertrophy. Interestingly, the *t*10, *c*12 CLA isomer also showed a consistent effect in that this isomer lowered kidney weight and attenuated MGV in *fa/fa* rats fed this isomer relative to *fa/fa* rats fed the CTL diet. The *t*10, *c*12 isomer also prevented an increase in the urine protein/creatinine ratio in both *fa/fa* and lean rats. This increase in protein/creatinine ratio was observed in rats fed the *c*9, *t*11 isomer or the combination of isomers (TOG) relative to rats fed the CTL diet. The *t*10, *c*12 CLA

isomer was also beneficial in improving oral glucose tolerance in the *fa/fa* Zucker rats as will be discussed in the following section.

Serum Glucose During Oral Glucose Tolerance Testing (OGTT)

A significant interaction between diet x genotype was found for area under the curve for serum glucose during an OGTT. Oral glucose tolerance was improved in *fa/fa* rats fed the TOG, or the 10,12 diet compared to CTL, but not in those fed the 9,11 isomer alone (**Figure 19**). The *fa/fa* rats also had impaired oral glucose tolerance compared to lean rats for all diets. The fact that the *fa/fa* rats displayed impaired glucose tolerance makes sense, as this rat model is hyperinsulinemic (by 3-4 weeks of age) and displays peripheral insulin resistance (Bray, 1977). It appears as though it is the *t*10, *c*12 CLA isomer that shows the beneficial effect on glucose tolerance, as only *fa/fa* rats fed this isomer alone, or together with the *c*9, *t*11 isomer, showed improvements in oral glucose tolerance. Therapeutic use of this isomer in humans should not be advocated based on these findings as it is the *t*10, *c*12 CLA isomer that has been shown to induce insulin resistance in males with the metabolic syndrome (Risérus et al., 2002a)

Houseknecht et al. (1998) reported that a 1.5% CLA mixture fed for 14 days improved glucose tolerance in the ZDF rat as determined by a glucose tolerance test with glucose injected intraperitoneally. A follow up study by this research group was conducted to look at isomer-specific properties of CLA in the ZDF rat model (Ryder et al., 2001). They found that feeding the *c*9, *t*11 isomer alone or feeding the control diet for 14 days, resulted in rats that were

hyperglycemic compared to lean controls as determined by postprandial blood glucose concentrations. However, rats that received the 50:50 isomer mixture (*c*9, *t*11 and *t*10, *c*12) and the pair-fed group maintained normal postprandial glycemia throughout the experiment. An intraperitoneal glucose tolerance test performed on day 11 revealed that glucose tolerance was improved in rats fed the 50:50 isomer mixture versus rats fed the control diet as determined by area under the curve analysis. Pair-feeding had an intermediate effect on glucose tolerance (Ryder et al., 2001). This study is in agreement with the current study in that they both demonstrate that the *t*10, *c*12 CLA isomer improves glucose tolerance in a diabetic rat model, as well as in a rat model of the metabolic syndrome, respectively.

When serum glucose from the OGTT was examined as repeated measures, a time x genotype x diet effect was present (**Figure 20**). The *fa/fa* rats fed the 10,12 diet had a higher serum glucose concentration compared to all other groups at t=0. This could indicate fasting hyperglycemia relative to rats fed the other diets. The *fa/fa* rats given the 9,11 and TOG diets had a lower serum glucose concentration compared to *fa/fa* rats fed the CTL diet at t=15. The 10,12 and the TOG diets lowered serum glucose concentration in the *fa/fa* rats compared to *fa/fa* rats fed the CTL or the 9,11 diet at t=30. Similarly the 10,12 diet and the TOG diet lowered serum glucose concentrations in *fa/fa* rats at t=60 compared to *fa/fa* rats fed the CTL diet. The 9,11 diet had an intermediate effect on serum glucose concentration at t=60. In summary, the diets that appeared to be most beneficial in improving oral glucose tolerance in the *fa/fa* Zucker rats

were the 10,12 and the TOG diets. It is therefore reasonable to say that it is the *t*10, *c*12 CLA isomer that is responsible for lowering serum glucose concentrations in this model.

The *fa/fa* rats had impaired oral glucose tolerance compared to lean rats at all time points ($P<0.0001$). The *fa/fa* rats displayed a fasting hyperglycemia (t=0) relative to lean rats. Hayashi et al. (2002) reported serum glucose concentrations in 14-16 week old *fa/fa* Zucker rats and lean rats to be 9.5 $\pm$ 1.2 and 6.9 $\pm$ 0.4 mmol/L, respectively, as determined at the time of termination. This corresponds with the concentrations found in this study at (t=0), which were 9.4 $\pm$ 0.3 and 7.6 $\pm$ 0.2 mmol/L for *fa/fa* and lean rats, respectively. In comparison, ZDF rats (14-15 weeks of age) have a fasting plasma glucose of approximately 8 mmol/L, and a non-fasted plasma glucose of greater than 20 mmol/L (Li et al., 2005). The *fa/fa* Zucker rats in this study displayed impaired glucose tolerance after oral administration of a glucose load. This impaired glucose tolerance can be seen at all time points following the oral glucose dose (t=15, t=30 and t=60) in the present study.

To summarize these findings, the 10,12 and the TOG diets were beneficial in attenuating oral glucose tolerance in *fa/fa* Zucker rats as determined by area under the curve for serum glucose. Despite a higher fasting serum glucose concentration in *fa/fa* rats fed the 10,12 diet, improved glucose tolerance occurred in the *fa/fa* rats fed the 10,12 diet compared to *fa/fa* rats fed the CTL diet by the mid point of the OGTT. At t=30 and t=60, the *fa/fa* rats fed the 10,12

or the TOG diet had lower serum glucose concentrations compared to *fa/fa* rats fed the CTL diet.

These results suggest a potential relationship between the improvements in oral glucose tolerance by the *t*10, *c*12 CLA isomer and improvements in renal disease progression by this isomer as measured by MGV.

Maddox et al. (2002) found that initiating feed restriction at 6 or 12 weeks of age in female *fa/fa* Zucker rats to the feed intakes of lean rats (14 g/day) reduced weight gain and prevented the development of glomerular hypertrophy. Feed restriction also slowed the development of hyperinsulinemia. Interestingly, the authors indicated that plasma glucose concentrations were similar throughout the study among groups but did not include this data. Based on these findings, it appears as though hyperinsulinemia is associated with renal injury in the *fa/fa* Zucker rat and that weight and insulin reduction seem to be involved in the attenuation of an enlarged MGV.

Protein Expression of Enzymes Involved in Eicosanoid Production

A genotype effect was present for cytosolic $cPLA_2$, in that *fa/fa* rats had a 35% higher protein expression for cytosolic $cPLA_2$ compared to lean rats (**Figure 21**). This is in contrast to what was reported by Warford (2003), where no significant effects were found for cytosolic $cPLA_2$. This enzyme is responsible for preferentially cleaving AA (over other unsaturated fatty acids) from membrane phospholipids, the first step in eicosanoid synthesis (Kramer & Sharp, 1997). When $cPLA_2$ is in the cytosol, it is in its inactive form. It is only when this enzyme

is stimulated by an increase in cytosolic free calcium (Ca^{2+}) that it becomes activated by binding to cellular membranes. More specifically, upon activation by Ca^{2+}, $cPLA_2$ binds to the endoplasmic reticulum and nuclear membranes (Kramer & Sharp, 1997). These results suggest that if the *fa/fa* rats have more $cPLA_2$ in the inactive form, there may be more enzyme available to become activated. It is therefore also important to look at the particulate fraction of this enzyme.

Surprisingly, in this study, there were no significant effects for particulate $cPLA_2$ (**Figure 22**). In contrast to this, the study by Warford (2003) demonstrated that *fa/fa* rats had higher protein levels of particulate $cPLA_2$ compared to lean rats, and Zucker rats (both lean and *fa/fa*) fed CLA had lower levels of particulate $cPLA_2$ compared to Zucker rats fed the control diet. The reason for the discrepancy between the 2 studies is not known. The diet effect may be explained by the fact that the diets differed between the two studies. These studies do demonstrate, however, that even if a difference exists in one fraction of $cPLA_2$ (cytosolic or particulate), this does not necessarily mean a difference will exist in the other fraction. A large amount of inactive enzyme does not guarantee a large amount of the activated form of that enzyme, as activation is not always concentration-dependant. The potential is there, but evidently this is not always the case.

The ratio of cytosolic $cPLA_2$ to particulate $cPLA_2$ was then calculated and it was found that the *fa/fa* rats had a higher cytosolic $cPLA_2$/particulate $cPLA_2$ ratio compared to the lean rats (**Figure 23**). This is different than what was found by Warford (2003) where lean rats fed CLA had a higher cytosolic

$cPLA_2$/particulate $cPLA_2$ ratio than any other group (lean rats fed control diet and *fa/fa* rats fed CLA or control diet). The results of the higher ratio of cytosolic $cPLA_2$/particulate $cPLA_2$ found in the present study confirms that *fa/fa* rats had a higher protein expression of the inactive form of $cPLA_2$ compared to lean rats, whether it was expressed on its own or as a ratio to particulate $cPLA_2$. The higher cytosolic $cPLA_2$ protein expression in the *fa/fa* rats may be associated with more advanced renal disease progression in this model. Even though the particulate fraction was not higher in *fa/fa* compared to lean rats, this does not mean enzyme activity of activated $cPLA_2$ was unaltered. Perhaps the *fa/fa* rats had a greater enzyme activity of particulate $cPLA_2$ that could then lead to a greater production of pro-inflammatory eicosanoids from AA. High levels of pro-inflammatory eicosanoids could act via a feedback mechanism to down regulate the amount of protein of the active form of $cPLA_2$ in the particulate fraction. Enzyme activity was not measured as it was beyond the scope of this thesis.

In this study, no significant effects were found for COX-1 (**Figure 24**). This agrees with the results found by Warford (2003).

The *fa/fa* rats fed the 10,12 diet had a lower COX-2 protein expression compared to *fa/fa* rats fed the CTL diet [pre-planned contrast (p=0.0198), **Figure 25**]. Since COX-2 is elevated in animal models of renal disease as shown in the present study and in other studies (Dey et al., 2004; Komers et al., 2005; Xu et al., 2005), having lower levels of this enzyme should prove beneficial in renal disease. It appears that in the current study, the *t*10, *c*12 CLA isomer was the specific isomer responsible for ameliorating the high COX-2 protein levels seen

in *fa/fa* rats. Warford (2003) did not find a significant effect of CLA on renal COX-2 protein expression, however, the numbers were changing in the same direction and were lower in both the *fa/fa* and lean Zucker rats fed CLA compared to *fa/fa* and lean rats fed the control diet, respectively.

COX-2 protein expression was elevated in *fa/fa* rats compared to lean rats for all diets (**Figure 25**). COX-2 expression was 552% higher in *fa/fa* rats fed the CTL diet compared to lean rats fed the CTL diet. Warford (2003) also found that COX-2 protein levels were higher in *fa/fa* compared to lean rats (187% higher). A higher COX-2 protein expression could lead to a higher production of downstream eicosanoids by this enzyme. These results are also consistent with those found by Dey et al. (2004) in the ZDF rat. These authors reported that COX-2 expression was increased 1.6 fold in the renal microvessels of obese 20-21 week old male rats, compared to lean rats (Dey et al., 2004). As well, Komers et al. (2005) recently found that COX-2 protein expression was higher in renal cortical tissue of male ZDF rats compared to lean littermate control rats at 4 weeks of age, and became even more prominent by 12 weeks of age. A recent study in the *fa/fa* Zucker rat concluded that COX-2 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 (Xu et al., 2005).

To summarize, the *t*10, *c*12 CLA isomer appears to be the isomer responsible for the beneficial effect in the kidneys of the *fa/fa* Zucker rat model of obesity and insulin-resistance. This isomer reduced MGV and prevented an increase in the protein/creatinine ratio. The *t*10, *c*12 isomer may mediate its

protective effects on the kidney by improving metabolic control and/or by altering eicosanoid production by COX-2.

The beneficial isomer in this study was the *t*10, *c*12 CLA isomer, not the *c*9, *t*11 CLA isomer found more abundantly in dairy products and ruminant meats. With the current state of knowledge, human supplementation with CLA for therapeutic use should not be recommended. The amount of CLA given to the rats in the current study relative to their body weight is quite a large dose, and would should not be taken by humans until long-term efficacy and safety studies consistently agree that CLA is not harmful.

CONCLUSION

In conclusion, dietary supplementation with the *t*10, *c*12 CLA isomer reduced MGV in 14 week old *fa/fa* Zucker rats compared to *fa/fa* rats fed the CTL diet. The *t*10, *c*12 isomer also prevented the development of elevated urine protein/creatinine in Zucker rats that was seen in rats fed the *c*9, *t*11 isomer or in rats fed both the *c*9, *t*11 and *t*10, *c*12 in combination (TOG). In addition, the *fa/fa* rats given the *t*10, *c*12 isomer alone or in combination with the *c*9, *t*11 isomer, demonstrated improved oral glucose tolerance compared to *fa/fa* rats given the CTL diet. As well, COX-2 protein levels were reduced only in *fa/fa* rats given the *t*10, *c*12 isomer alone compared to *fa/fa* rats given the CTL diet. This suggests that the *t*10, *c*12 CLA isomer may mediate its protective effects on early obesity-associated nephropathy by ameliorating metabolic control and/or altering eicosanoid production by COX-2.

STRENGTHS AND LIMITATIONS

Several limitations exist in the current study. One of these is that enzyme activity was not measured, so conclusions regarding enzyme activity of $cPLA_2$, COX-1 and COX-2 cannot be made. This is an important limitation as protein level does not always correspond with protein activity.

Secondly, this study was done using a rat model, so extrapolations as to what might occur in humans must be done with caution. Unfortunately, the nature of the experiment did not allow for a human clinical trial, so an animal model was chosen that best represented the human condition of interest.

Thirdly, body composition was not analyzed in these rats, which rendered some of the interpretations more difficult as mentioned in the discussion.

Another limitation is that only one dose of CLA was used in this study. Therefore, conclusions about whether or not higher CLA doses would result in greater improvements in kidney disease progression, as well as conclusions regarding the optimal dose of CLA in obesity-related kidney disease cannot be drawn. In the study by Warford (2003), a level of 1.5% dietary CLA was used for supplementation. The levels of the individual isomers used in the present study were comparable to the levels of the individual isomers in the study by Warford (2003). A higher total CLA level was used in the study by Warford (2003) due to the isomeric composition of the mixture.

A strong point of this study is that the individual CLA isomers were analyzed separately, as well as in combination. Many studies to date have used a mixture of CLA isomers, making it impossible to conclude which specific CLA isomer is responsible for which effects. The need for continued studies evaluating the properties of individual CLA isomers must be stressed.

Another strength of this study is that dietary intervention began early in the disease process. It is important to focus on prevention strategies of kidney disease, and one way to evaluate these strategies is by initiating them before the disease has significantly progressed.

FUTURE RESEARCH

Future research in this area should focus on:

- Gaining a better understanding on the mechanisms underlying kidney disease associated with the metabolic syndrome by continuing to evaluate eicosanoid production and function in obesity-associated renal disease
- Isomer-specific properties of CLA on enzyme activity of $cPLA_2$ by measuring AA release from membrane phospholipids
- Isomer-specific properties of CLA on enzyme activity of COX-1 and COX-2 by measuring eicosanoid production, namely PGI_2, PGE_2 and TXA_2
- Determine whether CLA displaces AA in phospholipids, affects COX-1 or COX-2 at the level of mRNA, protein or activity or acts as a substrate for COX enzymes
- Optimal dosing of specific CLA isomers in this model of obesity-associated renal disease
- Long-term studies to evaluate efficacy and safety of CLA isomer supplementation on renal disease progression
- Studies evaluating effectiveness of dairy/ruminant meat CLA isomers versus industrially produced CLA isomers

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APPENDIX

Table 6: Feed Intake and Body Weight								
	9,11	**10,12**	**TOG**	**CTRL**	**genotype**	**diet**	**g x d**	**contrast**
Total Feed Intake (g)								
fa/fa	1563^{ab} ± 55	1400^{c} ± 30	1539^{b} ± 50	1666^{a} ± 51				
lean	959^{d} ± 21	999^{d} ± 17	1023^{d} ± 23	1022^{d} ± 24	<0.0001	0.0033	0.0126	10,12 vs. CTRL 0.0049
Final Body Weight (g)								
fa/fa	543 ± 13	545 ± 10	567 ± 18	561 ± 13				
lean	336 ± 7	333 ± 9	327 ± 7	328 ± 5	<0.0001	0.9755	0.3871	

Mean ± SEM, n=10/group

Different letters indicate means are significantly different ($P<0.05$ for main effects and $P<0.10$ for interaction) as determined by Duncan's Multiple Range Test.

Table 7: Kidney Weight

	9,11	10,12	TOG	CTRL	genotype	diet	g x d	contrast
Kidney Weight (g)								
fa/fa	2.9^{ab} ± 0.1	2.8^{b} ± 0.1	2.9^{ab} ± 0.1	3.0^{a} ± 0.1				
lean	2.4^{c} ± 0.0	2.4^{c} ± 0.1	2.4^{c} ± 0.1	2.3^{c} ± 0.1	<0.0001	0.4376	0.2897	10,12 vs.CTRL 0.067
Kidney Weight/100 g Body Weight								
fa/fa	0.52^{b} ± 0.01	0.51^{b} ± 0.01	0.52^{b} ± 0.01	0.54^{b} ± 0.01				
lean	0.72^{a} ± 0.02	0.71^{a} ± 0.01	0.75^{a} ± 0.01	0.71^{a} ± 0.02	<0.0001	0.4283	0.1809	TOG vs.CTRL 0.0335

Mean ± SEM, n=10/group

Different letters indicate means are significantly different ($P<0.05$ for main effects and $P<0.10$ for interaction) as determined by Duncan's Multiple Range Test

Table 8: Urinary Parameters								
	9,11	10,12	TOG	CTRL	genotype	diet	g x d	contrast
Urine Volume (mL)								
fa/fa	5.7 ± 0.9	6.7 ± 1.1	7.7 ± 0.8	6.3 ± 1.3	0.0010	0.3930	0.8316	
lean	4.6 ± 0.4	4.3 ± 0.7	5.1 ± 0.9	3.8 ± 0.7				
Urinary Protein (mg/12 h)								
fa/fa	15.0 ± 2.3	15.0 ± 2.0	17.7 ± 3.3	8.1 ± 1.0	0.0002	0.1706	0.5868	
lean	9.9 ± 2.2	6.7 ± 0.7	9.2 ± 2.0	6.4 ± 1.1				
Urinary Creatinine (mg/12 h)								
fa/fa	3.1 ± 0.4	3.3 ± 0.3	3.5 ± 0.2	3.6 ± 0.5	<0.0001	0.8414	0.3650	
lean	5.2 ± 0.3	5.2 ± 0.5	4.4 ± 0.5	4.7 ± 0.2				
Urinary Protein/Creatinine (mg/mg)								
fa/fa	4.8 ± 0.6	4.5 ± 0.4	5.0 ± 0.8	3.0 ± 0.4	<0.0001	0.0447*	0.4672	
lean	1.9 ± 0.4	1.3 ± 0.1	2.0 ± 0.3	1.4 ± 0.2				

Urinary Glucose (µmol/12 h)

fa/fa	8.6 ± 3.0	5.5 ± 0.6	6.3 ± 0.5	7.7 ± 1.6	0.0004	0.8593	0.7140
lean	3.9 ± 0.3	4.5 ± 0.5	4.0 ± 0.5	4.3 ± 0.4			

* for Duncan's letters see Figure 12

Mean ± SEM, n=10/group (except Urinary Glucose where n=9 for *fa/fa* 9,11)

Different letters indicate means are significantly different ($P<0.05$ for main effects and $P<0.10$ for interaction) as determined by Duncan's Multiple Range Test.

Table 9: Serum Creatinine and Creatinine Clearance								
	9,11	**10,12**	**TOG**	**CTRL**	**genotype**	**diet**	**g x d**	**contrast**
Serum Creatinine (µmol/L)								
fa/fa	44.82 ± 2.12	47.21 ± 2.39	44.55 ± 2.56	41.99 ± 2.92	<0.0001	0.4722	0.7601	
lean	34.39 ± 1.24	33.42 ± 1.77	34.74 ± 1.68	32.18 ± 2.21				
Creatinine Clearance (mL/min)								
fa/fa	0.894 ± 0.133	0.887 ± 0.099	0.975 ± 0.080	1.104 ± 0.223	<0.0001	0.8631	0.5796	
lean	1.894 ± 0.129	2.021 ± 0.316	1.786 ± 0.097	1.884 ± 0.211				
Creatinine Clearance/100 g Body Weight (mL/min/100 g)								
fa/fa	0.177 ± 0.021	0.162 ± 0.018	0.172 ± 0.013	0.197 ± 0.039	<0.0001	0.7816	0.8466	
lean	0.561 ± 0.032	0.517 ± 0.025	0.552 ± 0.033	0.520 ± 0.034				

Mean ± SEM, n=10/group (except Creatinine Clearance/100 g Body Weight where n=9 for lean 10,12; lean TOG; lean CTL and *fa/fa* 9,11)

Table 10: Mean Glomerular Volume (MGV)								
	9,11	10,12	TOG	CTRL	genotype	diet	g x d	contrast
Mean Glomerular Volume ($\mu m^3 x 10^6$)								
fa/fa	$7.37x10^{6\ ab}$ $\pm 0.63x10^6$	$6.86x10^{6\ b}$ $\pm 0.31x10^6$	$7.42x10^{6\ ab}$ $\pm 0.51x10^6$	$8.54x10^{6\ a}$ $\pm 0.64x10^6$	<0.0001	0.5661	0.2909	10,12 vs. CTRL 0.0624
lean	$5.48x10^{6\ c}$ $\pm 0.35x10^6$	$5.57x10^{6\ c}$ $\pm 0.33x10^6$	$5.32x10^{6\ c}$ $\pm 0.29x10^6$	$5.34x10^{6\ c}$ $\pm 0.27x10^6$				

Mean ± SEM, n=10/group

Different letters indicate means are significantly different ($P<0.05$ for main effects and $P<0.10$ for interaction) as determined by Duncan's Multiple Range Test.

Table 11: Area Under the Curve (AUC) for Serum Glucose During an Oral Glucose Tolerance Test (OGTT)								
	9,11	**10,12**	**TOG**	**CTRL**	**genotype**	**diet**	**g x d**	**contrast**
AUC serum glucose (mmol*L^{-1}*min)								
fa/fa	958^{ab} ± 52	849^{bc} ± 54	784_{c} ± 35	1059^{a} ± 48	<0.0001	0.0052	0.0238	10,12 vs. CTRL 0.0531 TOG vs. CTL 0.0039 9,11 vs. TOG 0.0413
lean	611^{d} ± 26	594^{d} ± 16	616^{d} ± 36	622^{d} ± 25				

Mean ± SEM, n=10/group

Different letters indicate means are significantly different (P<0.05 for main effects and P<0.10 for interaction) as determined by Duncan's Multiple Range Test.

Table 12: Serum Glucose Concentrations at Various Time Points During an Oral Glucose Tolerance Test (OGTT)

	9,11	10,12	TOG	CTRL
Serum Glucose (mmol/L)*				
t=0 *fa/fa*	9.1^{b} ± 0.4	10.5^{a} ± 0.7	9.0^{bc} ± 0.2	9.1^{bc} ± 0.8
t=0 lean	8.0^{bcd} ± 0.4	7.7^{bcd} ± 0.5	7.1^{d} ± 0.3	7.6^{cd} ± 0.2
t=15 *fa/fa*	14.9^{b} ± 0.8	15.7^{ab} ± 0.9	14.4^{b} ± 0.7	17.4^{a} ± 1.1
t=15 lean	11.1^{c} ± 0.7	10.1^{c} ± 0.5	10.8^{c} ± 0.7	11.1^{c} ± 0.5
t=30 *fa/fa*	17.5^{a} ± 1.0	14.7^{b} ± 1.3	13.3^{cb} ± 0.7	18.6^{a} ± 1.1
t=30 lean	10.5^{d} ± 0.6	10.6^{d} ± 0.4	11.1^{cd} ± 0.9	10.8^{d} ± 0.5
t=60 *fa/fa*	18.3^{b} ± 1.3	13.7^{c} ± 1.0	13.4^{c} ± 0.7	20.7^{a} ± 1.2
t=60 lean	9.9^{d} ± 0.5	9.8^{d} ± 0.6	10.1^{d} ± 0.5	10.4^{d} ± 0.4

*time x genotype x diet P<0.0001

Mean ± SEM, n=10/group

Different letters indicate means are significantly different (P<0.05) as determined by Duncan's Multiple Range Test.

Table 13: Western Immunoblotting: Protein Optical Density								
	9,11	**10,12**	**TOG**	**CTRL**	**genotype**	**diet**	**g x d**	**contrast**
Cytosolic $cPLA_2$ (arbitrary units)								
fa/fa	0.346 ± 0.106	0.328 ± 0.095	0.272 ± 0.088	0.291 ± 0.064	0.0232	0.7172	0.9592	
lean	0.288 ± 0.078	0.187 ± 0.059	0.179 ± 0.063	0.250 ± 0.067				
Particulate $cPLA_2$ (arbitrary units)								
fa/fa	1.064 ± 0.078	0.757 ± 0.161	0.839 ± 0.173	0.778 ± 0.122	0.1293	0.8650	0.6389	
lean	0.994 ± 0.169	1.030 ± 0.171	1.027 ± 0.171	1.098 ± 0.224				
Ratio cytosolic $cPLA_2$/particulate $cPLA_2$ (arbitrary units)								
fa/fa	0.603 ± 0.277	0.368 ± 0.076	0.609 ± 0.243	0.484 ± 0.142	0.0090	0.8183	0.9703	
lean	0.350 ± 0.103	0.293 ± 0.116	0.173 ± 0.062	0.279 ± 0.088				
COX-1 (arbitrary units)								
fa/fa	1.224 ± 0.248	1.047 ± 0.443	0.676 ± 0.215	0.929 ± 0.230	0.3730	0.7191	0.6029	
lean	0.848 ± 0.153	0.919 ± 0.235	1.094 ± 0.282	1.261 ± 0.418				

COX-2 (arbitrary units)

fa/fa	1.751^{ab} ± 0.353	1.154^{b} ± 0.213	1.335^{ab} ± 0.273	1.885^{a} ± 0.324	<0.0001	0.8547	0.0991	10,12 vs.CTRL 0.0198
lean	0.309^{c} ± 0.073	0.445^{c} ± 0.068	0.350^{c} ± 0.072	0.292^{c} ± 0.052				

Mean ± SEM, n=9/group (except cytosolic $cPLA_2$ where n=8 for *fa/fa* 9,11; ratio of cytosolic $cPLA_2$/particulate $cPLA_2$ where n=8 for *fa/fa* 10,12; COX-1 where n=8 for *fa/fa* 10,12 and lean 10,12; n=7 for lean TOG and lean CTL and n=6 for lean 9,11)

Different letters indicate means are significantly different ($P<0.05$ for main effects and $P<0.10$ for interaction) as determined by Duncan's Multiple Range Test.