DIETARY CONJUGATED LINOLEIC ACID (CLA) REDUCES PROTEIN LEVEL OF CYTOSOLIC PHOPHOLIPASE A_2 AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA AND AMELIORATES EARLY RENAL DISEASE PROGRESSION IN OBESE *fa/fa* ZUCKER RATS

BY

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A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for a Degree of

MASTER OF SCIENCE

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Conjugated linoleic acid (CLA) alters rate-limiting enzymes involved in eicosanoid synthesis and slows the progression of early renal disease in the Han:SPRD-*cy* rat, a model of Polycystic Kidney Disease. CLA also is a ligand and activator of peroxisome proliferator-activated receptors (PPARs), which have been linked to the amelioration of early renal disease in animal models. Therefore, we examined the effects of dietary CLA on kidney function and morphology as well as protein levels of enzymes involved in eicosanoid synthesis and protein levels of PPAR isoforms in the kidneys of young obese *fa/fa* Zucker rats with early diabetic nephropathy. Six week old obese *fa/fa* Zucker rats (*fa*) and lean Zucker rats (*ln*) were fed 1.5% CLA by weight or the control diet (CTRL) for 8 weeks. The *fa* group had a 66% lower creatinine clearance and a 109% higher 12 hr urinary protein excretion than the *ln* group. The *fa* group also had 14% and 28% larger kidney weights and 30% and 84% larger mean glomerular volumes (MGVs) than the *ln* group fed CLA and CTRL, respectively. Feeding CLA to *fa* resulted in 11% smaller kidney weights and 28% smaller MGVs than feeding CTRL. The *fa* group had a 35% higher particulate cytosolic phospholipase A_2 ($cPLA_2$) protein level than the *ln* group and feeding CLA to Zucker rats overall resulted in a 26% lower particulate $cPLA_2$ protein level than feeding CTRL. The *fa* group also had a 187% higher cyclooxygenase 2 (COX-2) protein level than the *ln* group. The *fa* group had a 22% lower PPAR α and 31% higher PPAR β protein level than the *ln* group and feeding CLA to Zucker rats overall resulted in a 21% lower PPAR α protein level than feeding CTRL, but did not alter PPAR β. In conclusion, early feeding of CLA to *fa* results in smaller kidney weights and MGVs compared to those fed CTRL. Amelioration of these pathological signs of renal disease may be related to lower renal $cPLA_2$ and PPAR α protein levels in CLA fed rats.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ABSF	4-(2-aminoethyl) benzene sulfonyl fluoride
APS	ammonium persulfate
BCA	bicinchoninic acid
CLA	conjugated linoleic acid
COX	cyclooxygenase
CTRL	control
$cPLA_2$	cytosolic phospholipase A_2
DHA	docosahexaenoic acid
DN	diabetic nephropathy
EDTA	ethylene-diamine-tetraacetic acid
EETs	eicosatrienoic acids
EGTA	ethylene glycol-bis(β-amino ethyle ether) N,N,N',N'-tetracetic acid
EPA	eicosapentaenoic acid
ESRD	end stage renal disease
fa	*fa/fa* Zucker rats
FPW	foot process width
GFR	glomerular filtration rate
HETEs	hydroxy eicosatetraenoic acids
HPLC	high performance liquid chromatography
HPETEs	hydroperoxy eicosatetraenoic acids
IDV	integrated density value
ln	lean Zucker rats
LT	leukotrienes
MA	microalbuminuria
MAA	macroalbuminuria
MGA	mean glomerular area
MGV	mean glomerular volume
NA	normoalbuminuria
NSAIDs	non-steroidal anti-inflammatory drugs
OD	optical density
OHA	oral hypoglycemic agent
OLETF	Otsuka Long-Evans Tokushima fatty rat
PBS	phosphate buffered saline
PG	prostaglandin
PLA_2	phospholipase A_2
PPARs	peroxisome proliferator-activated receptors
PPREs	peroxisome proliferator response elements
PVDF	polyvinylidene fluoride
RRT	renal replacement therapy
RXR	9-cis-retinoic acid receptor
SDS	sodium dodecyl sulphate
T1	streptozotocin induced Type 1 diabetes mellitus
TBS	tris base solution
TCA	tricholoracetic acid

TEMED	tetramethylethylenediame
TX	thromboxane
TZD(s)	thiazolidinedione(s)
ZDF	Zucker diabetic fatty

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1 RATIONALE

Diabetic nephropathy (DN) is the number one cause of end-stage renal disease (ESRD) in Canada and the Western world, accounting for approximately 30% of all patients who have ESRD (Meltzer et al, 1998; Gall et al, 1993; Gall et al, 1991). It is anticipated that ESRD will rise 5.8% annually up to the year 2005 and that the number of Canadians living with diabetes by the year 2010 will be 50% greater than the year 1998 (Schaubel et al, 1999; Meltzer et al, 1998). Type 2 diabetes accounts for approximately 80%-90% of all diagnosed cases of diabetes and 10-20% of these individuals will ultimately develop ESRD, representing approximately 50% of all ESRD cases due to DN (Ibrahim et al, 1997; Gall et al, 1993; Gall et al, 1991).

Currently there is no cure for DN. However, many of the risk factors for this disease are potentially remediable via dietary intervention. Little research has focused on dietary intervention in the Type 2 diabetes patient with DN. Low protein diets, vegetable based protein diets and n-3 fats have shown beneficial effects in the attenuation of various renal diseases (Ogborn et al, 2003 – in press; Ogborn et al, 2002; Aukema et al, 2001; Ogborn et al, 1999; Anderson et al, 1998; Yanagisawa et al, 1998; Clark et al, 1995; Ingram et al, 1995; Tomobe et al, 1994; Yanagisawa et al, 1994; Aukema et al, 1992; Walker et al, 1989; Zatz et al, 1985). Recent investigations using conjugated linoleic acid (CLA) have shown that this naturally occurring fat is able to alter the production of eicosaniods and rate-limiting enzymes involved in eicosanoid production in a rat model of Polycystic Kidney Disease (unpublished data from Dr. Aukema's lab; Ogborn et al, 2003 – in press). In addition, CLA has been shown to slow the progression of this form of renal disease (Ogborn et al, 2003 – in press). Therefore, CLA may

ameliorate Polycystic Kidney Disease via alterations in eicosanoid production. Also newly discovered is that the renal production of eicosanoids is associated with the progression of early DN in a rat model of Type 2 diabetes and that Type 2 diabetes patients have significantly higher urinary levels of these eicosanoids compared to healthy individuals (Okumura et al, 2003; Hishinuma et al, 2001; Okumura et al, 2000).

Peroxisome proliferator-activated receptors (PPARs) are newly discovered, present in the kidney and have potential role(s) for the attenuation of DN (Guan et al, 2001; Guan and Breyer, 2001; Nicholas et al, 2001; Asano et al, 2000; Yang et al, 1999; Guan 1997; Braissant 1996). CLA has proven to be a ligand for PPAR α and PPAR γ (Moya-Camarena et al, 1999; Houseknecht et al, 1998). PPAR α regulates genes involved in lipid transport and lipid oxidation in the liver as well as anti-inflammation (Yurkova et al, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Devchand et al, 1996; Schoonjans et al, 1996). The lipid lowering effects of fibrates, also ligands for PPARs, are thought to be due to the activation of PPAR α (Fruchart et al, 1999; Ouali et al, 1998; Forman et al, 1997; Kliewer et al, 1997). Reduction of hyperlipidemia (with the use of fibrates) in the obese *fa/fa* Zucker rat, an animal model of obesity and the Type 2 diabetes "pre-diabetic state, has been shown to reduce proteinuria and glomerular injury (Kasiske et al, 1988).

PPAR γ is a key transcription factor involved in the terminal differentiation of adipocytes and regulates genes involved in anti-inflammation and glucose homeostasis (Houseknecht et al, 2002; Picard and Auwerz, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Gregoiré et al, 1998; Schoonjans et al, 1996). Troglitazone, a potent oral hypoglycemic agent (OHA) of the thiazolidinedione (TZD)

family and a ligand for PPAR γ, has been shown to ameliorate microalbuminuira (MA) in the streptozotocin-induced diabetes mellitus (T1) rat, a rat model of Type 1 diabetes, and Type 2 diabetes patients, as well as decrease proteinuria and halt renal structural changes typically seen in the obese *fa/fa* Zucker rat model (McCarthy et al, 2000; Imano et al, 1998; Fujii et al, 1997; Lehmann et al, 1995). One of the early structural changes observed in obese *fa/fa* Zucker rats is progressive glomerular hypertrophy after 10 weeks of age (Coimbra et al, 2000). CLA has been found to act similarly to troglitazone in terms of normalizing glucose tolerance, improving hyperinsulinemia and lowering circulating free fatty acids in the Zucker diabetic fatty (ZDF) rat via activation of PPAR γ (Houseknecht, 1998). The questions now elicited are can CLA act similar to fibrates and TZDs in terms of amelioration of DN?

Hypotheses: early CLA feeding to obese *fa/fa* Zucker rats, an animal model of obesity and the Type 2 diabetes "pre-diabetic" state will prevent deterioration in renal function and delay glomerular enlargement after 8 weeks of feeding. CLA will alter steady-state protein levels of rate-limiting enzymes involved in eicosanoid synthesis as well as eicosanoids themselves and alter the protein levels of PPARs in kidney tissue.

Objectives: To test these hypotheses, the kidneys of 6 week old obese *fa/fa* Zucker rats and lean Zucker rats fed either 1.5% CLA by weight or a control diet (CTRL) for 8 weeks will be analyzed for the following:

1) early histological change in the size of glomeruli via hemotoxylin and eosin staining of kidney cross-sections with subsequent measurement of mean glomerular volume (MGV)
2) steady-state renal levels of cyclooxygenase (COX)-1, COX-2, and cytosolic phospholipase A_2 ($cPLA_2$) protein using Western Immunoblotting
3) *ex vivo* renal production of prostaglandin (PG) E_2, PGI_2, and thromboxane (TX) A_2 using a competitive binding protein assay
4) steady-state renal levels of PPAR α, PPAR β, and PPAR γ protein using Western Immunoblotting

2 PRESENT STATE OF KNOWLEDGE

2.1 Type 2 Diabetes Mellitus

2.1.1 Description

Diabetes mellitus is defined as a metabolic disorder characterized by the presence of hyperglycemia due to defective insulin secretion, insulin action or both (Meltzer et al, 1998). There are three main types: Type 1, Type 2 and gestational. Type 1 diabetes is primarily the result of pancreatic beta-cell destruction. It includes all cases due to an autoimmune process and those for which the cause of beta-cell destruction is unknown. Type 2 diabetes can range from predominant insulin resistance with relative insulin deficiency to predominant insulin deficiency with insulin resistance. Gestational diabetes refers to glucose intolerance with onset during pregnancy.

2.1.2 Prevalence

Based on a 1996/97 Canadian survey and extrapolation from American data, the number of Canadians aged 12 and over with diabetes (diagnosed and undiagnosed) is estimated at 1.2 to 1.4 million (4.9% to 5.8% of this population), but excludes several areas of Canada including Indian reserves (Health Canada, 1999). Currently, First Nations children and adolescents of North Western Ontario and Manitoba have the highest reported prevalence of youth onset Type 2 diabetes in the world with children as young as age 8 being diagnosed (Dean et al, 1998; Sellers and Dean 1998). Type 2 diabetes accounts for approximately 80%-90% of all diagnosed cases of diabetes (Ibrahim and Hostetter, 1997).

2.1.3 Risk Factors

Diagnostic criteria for diabetes and glucose thresholds are summarized in Table 1. The factors that play a major role in the development of Type 2 diabetes include: 1) older age; 2) ethnicity (Aboriginal, Hispanic, Asian and African decent); 3) obesity; 4) a first-degree relative with diabetes; 5) a low level of high-density lipoprotein cholesterol ($\leq$ 0.9 mmol/L) or an elevated fasting level of triglycerides (> 2.8 mmol/L) (Meltzer et al, 1998). The 1998 clinical practice guidelines for the management of diabetes in Canada recommends that testing for diabetes using a fasting plasma glucose test should be performed every 3 years for those who are 45 years of age or older. As alluded to above, Type 2 diabetes is not just a disease of the older population, but is becoming more prevalent in the young. Due to the short history of this problem (since the early '80's) the most appropriate screening guidelines have not been developed (Meltzer et al, 1998). Earlier or more frequent testing should be considered for those who have additional risk factors for diabetes, as stated above. Annual testing should be considered for those with one or more of the following more predictive risk factors (irrespective of the above factors): 1) impaired glucose tolerance or impaired fasting glucose; 2) presence of hypertension; 3) presence of coronary artery disease; 4) a history of gestational diabetes; 5) presence of complications associated with diabetes (cardiovascular disease, hypertension, retinopathy, neuropathy and nephropathy).

Table 1 Diagnostic blood glucose levels (adapted from Meltzer et al, 1998 and Rose, 1995)

Category	FPG; mmol/L	PG 1 h after 75-g glucose load; mmol/L	PG 2 h after 75-g glucose load; mmol/L
Normal	3.3 – 6.0	8.9 – 10.6	<7.8
Impaired fasting glucose (IFG)	6.1- 6.9	N/A	N/A
Impaired Glucose Tolerance (IGT)	<7.0	N/A	7.8-11.0
Diabetes Mellitus (DM)	≥7.0	N/A	≥11.1*
Gestational diabetes mellitus (GDM)**	≥5.3	≥10.6	≥8.9

FPG = fasting plasma glucose, PG = plasma glucose, NA = not applicable
*Requires symptoms of diabetes to be present if it is a casual blood test. Symptoms may include: fatigue, polyuria, polydipsia, unexplained weight loss.
**Requires 2 abnormal values among the three.

2.1.4 Complications

Long-term complications of diabetes mellitus can be classified as microvascular or macrovascular diseases (Franz, 1996). Cardiovascular disease and hypertension are classified as macrovascular complications of diabetes mellitus while retinopathy, neuropathy and nephropathy are classified as microvascular complications of diabetes mellitus.

2.2 Diabetic Nephropathy

2.2.1 Description

As stated above, nephopathy is a complication of diabetes mellitus. In a review by Parving et al in 1996, DN is defined as a clinical syndrome characterized by persistent albuminuria (> 300 mg/24 hr), a continuous decline in glomerular filtration rate (GFR), and raised arterial blood pressure. It involves a progressive worsening of kidney function as characterized by specific renal lesions and clinical manifestations in the patient with diabetes. The stages of DN are often clinically marked according to the degree of proteinuria. In 1987 Mogensen et al concluded that microalbuminuria (MA) leads to overt nephropathy in 80% of young Type 1 patients and 22% of elder Type 2 patients after a 10 year follow-up. In this paper Mogensen et al defines MA as urinary albumin excretion > 20 µg/min but ≤ 200 µg/min or 30 to 300 mg/24 hr.

Mogensen et al (1987) also proposes various developmental stages of DN. Please see Table 2 for a summary of these stages. Stage I is designated as glomerular hyperfunction and hypertrophy. Stage II, designated the “silent” stage, is characterized by development of renal lesions, which are predominantly in the glomerulus, with normal

Table 2 Progression of diabetic nephropathy (adapted from Gillis, 1998 and Mogensen et al, 1987)

Stage	Designation	Characteristics	GRF (mL/min)	Albumin Excretion	Blood Pressure
Stage I	Hyperfunction and Hypertrophy	Glomerular Hyperfiltration and Hypertrophy	Increased in Type 1 and Type 2 diabetes	May be increased	Type 1 – normal Type 2 – normal or hypertension
Stage II	"Silent" Stage	Increasing BM and Expanding Mesangium	Normal	Type 1 – normal Type 2 – may be <30 – 300 mg/24 hr	Type 1 – normal Type 2 – normal or hypertension
Stage III	Incipient Diabetes	Between stages 2 and 4	Begins to fall	30 – 300 mg/24 hr (Microalbuminuria)	Type 1 – hypertension Type 2 – normal or hypertension
Stage IV	Overt Diabetic Nephropathy	Increasing rate of glomerular closure. Hypertrophy of remaining glomeruli.	Below normal	>300 mg/24 hr (Macroalbuminuria)	Hypertension
Stage V	Uremia	ESRD	0-10	Decreasing	Hypertension

albumin excretion rate. Stage III, designated "incipient DN", is characterized by MA, and causes increased risk of overt DN. Incipient DN is considered present when MA is found in 2 of 3 urine samples collected consecutively within a 6 month period. Stage IV, designated overt (clinical) DN, is characterized by macroalbuminuria (MAA), whereurinary protein excretion is > 200 μg/min or > 300 mg/24 hr, hypertension and a subsequent fall in GFR. Overt DN is considered present when urinary albumin excretion is > 300 mg/24 hr in at least 2 or 3 urine samples, collected within 6 months. Stage V is characterized by ESRD with uremia (excess urea and other nitrogenous waste in the blood). It should be noted here that normoalbuminuria (NA) is often defined by researchers as urinary albumin excretion ≤ 30 mg/24 hr, MA as 31-299 mg/24 hr and MAA as ≥ 300 mg/24 hr. In 1989 Humphrey et al reported that the average interval between the on set of proteinuria to the development of ESRD in Type 2 diabetes patients is 7 years.

Documented as far back as 1935, Kimmelstiel and Wilson found an association between glomerularopathy and Type 2 diabetes. However, the majority of studies on renal morphology have focused on the patient with Type 1 diabetes. As can be found in the review by Vestra et al in 2000, glomerulopathy in the Type 1 DM patient is characterized by thickening of glomerular basement membrane and mesangial expansion, leading to the progressive reduction in filtration surface of the glomerulus. Morphological changes also develop in the arterioles, tubules and interstitium. Although these pathological findings can also be found in other renal diseases, a group of fixed lesions, which include thickening of glomerular and tubular basement membranes,

mesangial expansion, Kimmelstiel-Wilson nodules, and arteriolar hyalinosis, is unique to DN and can be separated from all other renal disorders.

Bjørn et al (1995) have also found that the foot process width (FPW) of podocytes on peripheral basement membranes is increased in MA in Type 1 diabetes patients as compared to NA patients, but did not significantly correlate with the level of albumin excretion. The width of filtration slits was found to be greater in NA and MA patients with diabetes compared to those without diabetes (control). Patients with MAA had narrower filtration slits (similar to control) than those with MA. In all of the diabetes patients, the width of the filtration slits positively correlated with GFR. Thus, it was concluded that changes in podocyte and filtration slit width in Type 1 DM patients with nephropathy occur at the MA stage.

In Type 2 diabetes, the renal morphology is much more heterogeneous. In 1993, Østerby et al found that Caucasian Type 2 diabetes patients with proteinuria demonstrated the characteristic changes of DN seen in Type 1 diabetes patients with the same degree of proteinuria over an approximately 5.5 year time period. Structural changes included increased glomerular basement membrane thickness and mesangial volume and matrix volume (expressed as a fraction of the glomerular volume). Glomerular volume was larger in Type 2 diabetes patients compared to Type 1 diabetes patients and controls. Type 2 diabetes patients showed much more variation in structural parameters compared to the Type 1 diabetes group. Although there were no significant differences between the two groups, Type 2 diabetes patients had structural parameters that were closer to "normal" as compared to the Type 1 diabetes patients with the same degree of proteinuria. GFR was significantly lower in Type 1 diabetes patients compared

to the Type 2 group. Østerby et al (1993) described the coexistence of proteinuira and preserved GFR in Type 2 diabetes patients as a notable discrepancy between the two types of diabetes. This observation has also been noted by Moriya et al in 2002 and Fioretto et al in 1997. Østerby et al (1993) hypothesized that there may be some abnormalities in arteriolar structure that may lead to impaired vascular response to changes in systemic blood pressure; therefore, contributing to increased leakiness of the glomerular filter. Higher systolic and diastolic blood pressure was associated with more severe glomeruli involvement in Type 2 diabetes patients. The severity of glomerulopathy positively correlated with the rate of decline in GFR as it did for Type 1 diabetes patients. In both groups, the observed structural changes began with increased basement membrane thickness, which paralleled mesangial matrix expansion. However, in advanced glomerulopathy, predominance of matrix expansion is seen. With increasing glomerulopathy a smaller part of the blood stream borders the filtration barrier, which is likely to lower the GFR.

Similar findings have been reviewed by Vestra et al in 2000, which supports that in Type 2 diabetes the renal morphology is much more heterogeneous. In 1998, Fioretto et al found that several Caucasian Type 2 DM patients with persistent MA or MAA had normal glomerular structure, even though glomerular structure parameters were more altered on average from NA to MA and MAA. In addition, GFR was inversely related to mesangial fractional volume (expressed as a fraction of glomerular volume) but not to glomerular basement membrane width. Unlike Østerby et al (1993), Fioretto et al (1998) found that proteinuria did directly relate to structural parameters, such as glomerular

basement membrane width and mesangial fractional volume, although the relationship was less precise than in Type 1 diabetes.

In 1997 Pagtalunan et al found that broadening of podocyte foot processes in Type 2 diabetes Pima Indians was associated with a reduction in the number of podocytes per glomerulus and an increase in the surface area covered by remaining podocytes, suggesting that podocyte loss contributes to the progression of DN in the Type 2 diabetes as has been suggested for the Type 1 diabetes. Similar findings were concluded by Meyer et al in 1999.

2.2.2 Prevalence

DN is the number one cause of ESRD in Canada. (Meltzer et al, 1998). In 1999, the Canadian Organ Replacement Register, managed by the Canadian Institute of Health Information, reported that the percentage of individuals with newly diagnosed kidney failure who also have diabetes mellitus rose from 16% in 1981 to 28% in 1996. Type 2 diabetes accounts for approximately 80%-90% of all diagnosed cases of diabetes and 10-20% of these individuals will ultimately develop ESRD, representing approximately 50% of all ESRD cases with DN (Ibrahim and Hostetter, 1997). One of the reasons why only 10-20% of Type 2 diabetes patients develop ESRD may be because those with Type 2 diabetes have a predisposition to cardiac disease, which may lead to death before renal failure prevails. Renal disease appears at approximately the same time as cardiovascular disease in Type 1 diabetes patients, therefore, allowing a greater fraction of this group to arrive at ESRD (reviewed by Fioretto et al in 1997; Inbrahim and Hostetter in 1997; Østerby et al in 1993). However, with medical advancements in the prevention and

treatment of cardiovascular disease it may come as no surprise that Schaubel et al (1999) have projected large increases in ESRD incidence and prevalence, particularly among people with diabetes, as it is estimated that by the end of 2005 the number of people requiring renal replacement therapy (RRT) will increase by 85% from 1996 with a mean annual increase of 5.8%.

With the rise in incidence of youth onset Type 2 diabetes and with a greater number of adults living longer with Type 2 diabetes, research in the area of prevention and treatment of DN is becoming increasingly important.

2.2.3 Human and Economical Costs

The life of a patient with DN involves careful attention to dietary choices in order to control for protein, phosphorus, sodium, potassium, and fluid intake as the kidneys slowly lose their ability to excrete metabolic waste from protein or maintain electrolyte and fluid balance (American Dietetic Association, 2000). As renal function diminishes and the kidney has increasing difficulty excreting urea, fatigue and decreased mental acuity become obvious as uremia sets in (Merch Research Laboratories, 1992). Uremia can lead to peripheral neuropathies, muscle cramps and convulsions. Anorexia, nausea, vomiting, and an unpleasant taste in the mouth also are often present. Malnutrition is a common feature of chronic uremia. With advanced progression of the disease, gastrointestinal ulceration and bleeding are common.

There is no cure for ESRD, therefore, if the individual with DN progresses to this stage they are faced with one of two choices for RRT: 1) life long dialysis or 2) kidney

transplantation. As reviewed by Hutchinson in 1999, the cost to keep one patient with ESRD alive is estimated to be ~$75,000 annually.

2.2.4 Risk Factors

Ritz and Orth (1999) summarized the factors associated with an increase in the risk of progression to ESRD in Type 2 DM as the following: 1) elevated blood pressure; 2) albuminuria or proteinuria; 3) poor glycemic control (high insulin resistance), 4) smoking; 5) high dietary intake of protein; 6) hyperlipidemia, 7) family history of DN or risk factors of DN.

In 1998, Ravid et al published a study that involved 574 patients who were followed for 2 to 9 years and had recent onset of Type 2 diabetes, were normotensive and had normal albuminuria at baseline, in order to assess predictors for the progression of DN. A hemoglobin $A_{1c} \geq 0.09$, total cholesterol ≥ 5.24 mmol/L (203 mg/dL) and a mean blood pressure (diastolic pressure plus one third of the pulse pressure) ≥95 mm Hg when present together define patients at the highest risk of DN. Male sex, presence of smoking, a body mass index ≥ 25, high-density lipoprotein < 1.14 mmol/L (44 mg/dL) and low-density lipoprotein ≥ 3.21 mmol/L (124 mg/dL) at baseline all significantly increased the odds of developing MA.

In 1997, Yokoyama et al published a study that involved 182 Type 2 diabetes patients, who were followed for 8 years and who had a baseline serum creatinine of 133 μmol/L and diabetic proliferative retinopathy in order to assess predictors for the progression of DN. Presence at baseline of elevated systolic and diastolic blood pressure as well as elevated urinary protein excretion significantly and positively correlated with

the rate of decline of renal function. There was a significant inverse relationship with serum albumin and rate of decline of renal function. Presence, at baseline, of smoking, low serum albumin, family history of hypertension, increased urinary protein excretion, elevated total cholesterol and triglycerides as well as elevated platelet count significantly and positively correlated with the development of ESRD requiring dialysis. If adjusted for urinary protein excretion only family history of hypertension, serum albumin, diastolic blood pressure and smoking remained significant.

The risk factors for diabetic nephropathy in Type 2 diabetes are very relevant to the field of nutrition and disease research as many of these factors are potentially remediable via dietary intervention. Most of the research conducted in the area of human and animal models of renal disease has focused on dietary protein and fat intervention.

2.3 Dietary Intervention

2.3.1 Protein

Like the study of morphological changes of the kidney in DN the majority of studies on diet and DN have focused on the patient with Type 1 diabetes or other renal diseases. In 1988 Klahr et al reviewed the effects of dietary protein intake on the progression of various renal diseases and concluded that dietary protein restriction may be benefical in certain types of renal disease. In 1985 Zatz et al found that T1 rats with persistent moderate hyperglycemia who were fed a diet of 50% protein (wt/wt) developed higher GFRs, glomerular plasma flow and transcapillary hydraulic pressure compared to rats fed lower protein diets (6 or 12%). Those fed the high protein diets also developed large increases in urinary albumin excretion with time (200 mg/24 hr by one year) and

had a high incidence of glomerular lesions with 20% of glomeruli showing significant sclerosis, which correlated with prominent expansion of the mesangial area. Those fed the low protein diets had only low-grade albmuniuria and minimal glomerular sclerosis.

In 1989, Walker et al found that a low protein diet (40 g protein per day, half from animal sources and half from plant sources) attenuated the rate of decline of GFR in Type 1 patients with DN independently of blood pressure and changes in glycemic control. Similar findings were obtained in nephrectomized rats and it has been suggested the mechanisms involved with high dietary protein intake and disease progression may involve increased glomerular transcapillary hydraulic pressure and glomerular plasma flow rate as well as defective size and charge selective properties of the glomerular capillaries (Olson et al, 1982; Hostetter et al, 1981). A meta-analysis in 1996 by Pedrini et al also concluded that dietary protein restriction effectively slows the progression of DN in Type 1 patients.

In 1991, Jameel et al published one of the very few studies that investigated dietary protein intake as it relates to clinical proteinuria in Type 2 diabetes patients. Patient's duration of diabetes ranged from 4.7 to 10.4 years and included male and female subjects. Data was collected in two phases on two separate groups of subjects. In phase one (lasting 3 years), dietary protein intake was assessed using dietary recalls and in phase 2 (lasting 4 years) using a food-frequency questionnaire. Patients were divided into proteinuria categories as negative or trace versus ≥ 1. An albuminuria value of ≥ 30 mg/L was considered positive. No significant correlation between dietary protein intake and clinical proteinuria was observed after adjusting for age, sex, ethnicity, systolic blood pressure and 2-hour blood glucose.

Another article was published in 1999 by Pijls et al who were one of the first to look at the long-term effects (12 months) of protein restriction on albuminuria in patients with Type 2 diabetes. The study included 121 subjects with Type 2 diabetes who, at baseline, had MA or at least relatively high albuminuria within the normoalbuminuric range (albuminuria > 20 mg/24 hr in at least one sample or urinary albumin concentration > 6.5 mg/L in at least two samples) or diabetes duration ≥ 5 years. Patients were excluded if their baseline protein intake was < 0.80 g/kg/day. All participants received counseling from dietitians at the start of the study and every 3 months thereafter in order to decrease dietary protein intake to 0.8 g/kg/day by partially replacing protein, isocalorically, with unsaturated fat and carbohydrates. Dietary protein intake was estimated from 24-hr urinary urea excretion and from a semi-quantitative food-frequency questionnaire. Albuminuria was measured in duplicate 24-hr urine samples at baseline and after 6 and 12 months. In the experimental group, after 6 and 12 months, only 10% and 12% of participants, respectively, reached a protein intake of 0.80 g/kg/day; 16% and 19% of participants, respectively, showed a decrease of 0.20 g/kg/day or more. Plant protein intake was not decreased, while animal protein was decreased. Albuminuria was 28% lower in the experimental group compared to the control at 6 months, while at 12 months it was 18% lower. At six months, a 0.10 g/kg/day decrease in animal protein intake was related to an 11.1% decrease in albuminuria. Therefore, even though decreasing protein intake may be difficult, small changes may be beneficial.

In a 1999 review, Anderson et al suggested that not only does a low protein diet slow the progression of DN but the type of protein also may play an important role. Anderson et al, in 1998, studied the effects of soy protein on renal function and

proteinuria in patients with Type 2 diabetes. They proposed the soy-protein hypothesis, which states that substituting soy protein for animal protein in diabetes patients results in protection from and/or slowing of DN. This hypothesis was derived from studies carried out on nephrectomized rats, which showed soy protein had beneficial effects, and human studies, which suggested the source of protein may affect urine albumin excretion in patients with DN. The researchers reported that eating 50% of dietary protein as soy protein for 8 weeks had no distinct effect on renal function or proteinuria in these patients, but the diet was associated with a significant reduction in serum cholesterol and triglycerides.

Kontessis et al, in 1990, found that feeding healthy individuals vegetable based protein diets for 3 weeks had no significant effects on GFR, renal vascular resistance or renal plasma flow. However, when fed animal protein, GFR and renal plasma flow rose and renal vascular resistance fell. Differences are thought to be attributed to the different amino acid compositions of the two types of protein which may alter the glucagon and renal vasodilatory PG, particularly PGI_2, response. The response of both of these hormones was higher for the animal based protein diet and was significantly blunted by the vegetable protein.

Further studies have suggested these hemodynamic alterations may be mediated by PGs (Yanagisawa et al, 1994; Krishna et al, 1988; Stahl et al, 1987). Yanagisawa et al (1994) found the glomerular eicosanoids PGE_2, 6-keto $PGF_{1\alpha}$ (the stable metabolite of PGI_2) and TXB_2 (stable metabolite of TXA_2) and steady-state levels of COX were significantly increased in rats with bilateral ureteral obstruction who were fed a high

protein (40% casein) versus a low protein (6% casein) diet. These values fell markedly when the rats were pre-treated with the angiotensin converting enzyme inhibitor enalaprilat *in vivo*, thus suggesting that dietary protein affects increased glomerular eicosanoid production by altering the COX pathway, which is responsible for eicosanoid production via the renin-angiotensin-aldosterone system. Increased production of eicosanoids by glomeruli isolated from rats with bilateral ureteral obstruction has been shown to be mediated by angiotensin II-induced increases in COX activity via *de novo* synthesis of the enzyme (Yanagisawa et al, 1991; Yanagisawa et al, 1990).

In 1998, Yanagisawa and Wada also found that the synthesis of PGE_2, 6-keto $PGF_{\alpha 1}$ and TXB_2 were significantly increased in the medullary tubules of rats fed a high (40% casein) vs. low (6% casein) protein diet. This corresponded with greater activity of membrane associated PLA_2 and COX enzyme activity. Similar findings were reported by Yanagisawa et al (1992) in the glomeruli of rats.

Intervention involving dietary fat has also been shown to alter eicosanoid levels and attenuate various froms of renal disease, as will be discussed in the following section.

2.3.2 Fat

Research in the area of animal models of Polycystic Kidney Disease and human Lupus Erythematosus Nephritis have reported beneficial effects of flax oil, a rich source of the n-3 fatty acid α-linolenic acid, in the attenuation of these disease processes (Ogborn et al, 2002; Ogborn et al, 1999; Clark et al, 1995). In 1995, Ingram et al studied the affect of feeding dietary flax oil at 15% (wt/wt) for 20 weeks in the 5/6 renal ablation model. In this model, the right kidney is removed and two of the three vessels supplying

the left kidney are doubly ligated. The model represents a final common pathway to renal failure as observed in human diseases characterized by progressive loss of nephron function. Flax oil retarded the progression of renal injury as seen by the attenuation of the following, compared to those fed regular lab diet (not significant): decreased GFR, increased glomerularsclerosis and increased mesangial expansion. There was a significant attenuation of increased proteinuria compared to the regular lab diet. Flax oil also significantly prevented an increase in blood pressure after 20 weeks compared to the regular lab diet. The attenuation of renal injury correlated with a larger decline from pre-surgery in urinary 6-keto $PGF_{1\alpha}$ compared to feeding regular lab diet. The decrease in urinary 6-keto $PGF_{1\alpha}$ from pre-surgery in the two diets was significant, but the urinary 6-keto $PGF_{1\alpha}$ level for the regular lab diet versus flax oil was not significant. There was also a decline from pre-surgery in urinary TXB_2, which was significantly different than pre-surgery and regular lab diet levels. TXB_2 levels were increased from pre-surgery in rats fed regular lab diet. Overall, the ratio of urinary 6-keto $PGF_{1\alpha}$ to TXB_2 was decreased from pre-surgery in the regular lab diet group while feeding flax oil prevented this decline. The prevention of a decreased ratio was due to the prevention of an increased urinary TXB_2 by the flax oil diet. In 2002, Horiba et al found that renal cortical COX-2 and PGI_2 levels are up-regulated in the 5/6 renal ablation model.

In 1990, Sinha et al studied the effects of dietary fish oil on glomerular function in T1 rats. For a period of 20 weeks, T1 rats and normal rats were fed either a 20% (wt/wt) menhaden oil diet, which is a rich source of eicosapentaenoic acid (EPA) and other n-3 fatty acids or a 20% (wt/wt) beef tallow diet, which is rich in saturated fatty acids. Control rats without diabetes were given regular Purina rat chow. T1 rats had higher

GFRs upon initiation of the diet and at the end of the study period, as compared to normal rats. Dietary fat had no effect on GFR in T1 or normal rats. Menhaden oil did significantly reduce 25 hr urinary protein excretion in T1 rats despite no change in GFR. Eicosanoid synthesis was evaluated at the end of the study by incubating isolated glomeruli from the different experimental groups. Normal and T1 rats fed the beef tallow diet had comparable PGE_2 and TXB_2 levels. Levels of $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ in T1 rats fed beef tallow were slightly higher than normal rats feed beef tallow. Feeding menhaden oil significantly reduced the production of PGE_2, TXB_2, $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ by 50-60% in glomeruli of normal and T1 rats compared to normal and T1 rats fed beef tallow. At the end of the study period, none of the T1 rats exhibited overt renal changes as assessed by mesangial expansion or basement membrane thickness. In addition, feeding T1 rats menhaden oil significantly attenuated the rise in plasma triglycerides seen in the T1 rats feed beef tallow such that the triglyceride levels seen in the T1 rats fed menhaden oil was very similar to triglyceride levels found in normal rats fed Purina rat chow. Plasma cholesterol levels in T1 rats fed beef tallow were higher than normal rats fed Purina rat chow. Plasma cholesterol levels in T1 rats fed menhaden oil were lower than T1 rats fed beef tallow and normal rats fed Purina rat chow. The researchers concluded that enhanced glomerular eicosanoid production is not a major contributing factor in hyperfiltration in this rat model of Type 1 diabetes and that decreased eicosanoid production may play a role in attenuating urinary protein excretion.

A similar study was conducted by Wheeler et al in 1991 using 40 obese *fa/fa* Zucker rats that had undergone unilateral nephrectomy at 10 weeks of age. Unilateral nephrectomy was performed in order to exacerbate lipid abnormalities and accelerate the

onset or proteinuria. Rats were divided into one of three groups. One group received the control diet while the other two groups received a diet supplemented with either 14% fish oil or 14% melted beef tallow. Rats were held on this diet for 32 weeks post-nephrectomy or until 42 weeks of age at which time the animals were sacrificed and their kidneys removed for histological analysis and eicosanoid production assessment. The fish oil fed obese *fa/fa* Zucker rats had significantly lower mean plasma cholesterol and triglyceride levels and developed less proteinuria than control or beef tallow fed animals. PGE_2 and the stable metabolites of TXA_2 and PGI_2 were all significantly lower in the fish oil fed group compared to the beef tallow fed group. In relation to this study, in 1991, Kasiske et al revealed that the type of dietary fatty acid consumed could alter the fatty acid profile in the kidney of obese *fa/fa* Zucker rats. It was found that after 24 weeks of feeding, 34 week old obese *fa/fa* Zucker rats fed a 25% fish oil diet had higher n-3 polyunsaturated fatty acid kidney content as reflected by increased EPA and docosahexaenoic acid (DHA) levels while arachadonic acid (AA) levels were decreased. Obese *fa/fa* Zucker rats fed 25% sunflower oil had higher n-6 polyunsaturated fatty acid kidney content as reflected by increased linoleic acid while EPA levels were decreased. The degree of mesangial matrix expansion was lowest in rats fed sunflower oil while both the fish oil and sunflower oil groups had comparable reductions in glomerularsclerosis. Eicosanoid levels were not measured.

Recently, the renal production of the eicosanoids PGI_2 and TXA_2 have been associated with the progression of early DN in a rat model of Type 2 diabetes, the Otsuka Long-Evans Tokushima Fatty (OLETF) rat (Okumura et al, 2000). In addition, it has been found that in patients with Type 2 diabetes the urinary ratio of TXA_2 to PGI_2 (as

measured by their urinary metabolites 11-dehydro-thromboxane B_2 and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, respectively) is significantly higher than healthy individuals due to higher TXA_2 and lower PGI_2. (Hishinuma et al, 2001). In the Okumura et al (2000) study, beginning at 14 weeks of age, 24-hr urinary excretion of protein in the OLETF rats was similar to normal rats. By 30 weeks of age, urinary excretion of protein was significantly greater in the OLETF rats compared to normal rats at 30 weeks and compared to OLETF and normal rats at 14 weeks. Urinary protein excretion steadily increased in the OLETF rats up to 52 weeks, at which time the study was concluded. Urinary excretion of TXB_2 and 6-keto $PGF_{1\alpha}$ in OLETF rats was significantly higher beginning at 14 weeks compared to normal rats. Urinary TXB_2 peaked at 30 weeks of age and remained steady and significantly higher than normal rats until 54 weeks. Urinary 6-keto $PGF_{1\alpha}$ decreased slightly at 30 weeks of age and remained steady up to 54 weeks at which time values were similar to normal rats due to the peak of urinary 6-keto $PGF_{1\alpha}$ in normal rats at 54 weeks of age. Creatinine clearance, a measure of renal function, in OLETF rats at 14 weeks of age was not significantly different from normal rats. At 54 weeks of age OLETF rats had a creatinine clearance that was significantly lower than normal rats, indicating renal insufficiency. In summary, renal synthesis of PGI_2 and TXA_2 were increased at 14 weeks of age before proteinuria became marked, and as nephropathy progressed, a high level of TXA_2 synthesis was maintained while PGI_2 synthesis declined, thus decreasing the PGI_2 to TXA_2 ratio.

In 2003, Okumura et al published a follow-up study to investigate the role of TXA_2 in nephropathy in the OLETF rat model of Type 2 diabetes. The previous study measured urinary TXB_2 levels, a metabolite of TXA_2 in the kidney, using an antibody

that cross-reacted 60.5% with 2,3-dinor-TXB_2, a product of TXB_2 metabolism in the blood. Therefore, previous results may have included a large portion of 2,3-dinor-TXB_2 immunoreactivity thus skewing the data. The latest study consisted of three experimental groups which included normal controls, OLETF rats and OLETF rats treated with a daily dose of a TXA_2 synthase inhibitor starting at 10 weeks of age. At 62 weeks of age rats were terminated. The extent of glomerulosclerosis and thrombi in glomerular capillaries was greater in OLETF rats compared to OLETF rats treated with TXA_2 synthase inhibitor. Control rats had no glomerulosclerosis or thrombi. It was found that at 26 weeks of age and later, the administration of a TXA_2 synthase inhibitor decreased the severity of proteinuira in OLETF rats. Similar to findings in their previous study, OLETF rats had higher urinary TXB_2 than control rats starting at 14 weeks and remained steady thereafter. The TXA_2 synthase inhibitor decreased TXB_2 excretion. Starting at approximately 26 weeks of age, rats treated with the TXA_2 synthase inhibitor had about the same urinary TXB_2 levels as controls. An increase in urinary 2,3-dinor-TXB_2 in the OLETF rats was seen starting 26 weeks, the same time proteinuria appeared and was significantly higher than OLETF rats treated with the TXA_2 synthase inhibitor. Urinary 2,3-dinor-TXB_2 levels in OLETF rats increased in parallel with the progression of proteinuria and the TXA_2 synthase inhibitor inhibited this increase. Cross-reactivity of the 2,3-dinor-TXB_2 antibody with TXB_2 was 22.7%, therefore, results obtained with 2,3-dinor-TXB_2 would be expected to be more reliable. The researchers concluded that TXA_2 from platelets increases as Type 2 diabetes progresses and inhibition of platelet aggregation by a TXA_2 synthase inhibitor ameliorates the progression of DN.

The mechanism by which dietary fat is able to alter eicosanoid production is a key factor in the possible mechanism by which dietary fat may be able to ameliorate renal disease.

2.3 Eicosanoids

The potential mechanism involved in the smaller decline in urinary PGI_2 to TXA_2 ratio with the introduction of the α-linolenic rich flax oil in the 5/6 renal ablation model discussed above (Ingram et al, 1995) may, in part, be due to increased competition among the fatty acid precursors for entry into the various eicosanoid forming pathways. In order to understand this concept, one must first understand how eicosanoids are formed.

The word "eicosanoids" refers to all the oxygenated products synthesized from 20-carbon polyunsaturated fatty acids by the COX pathway, as well as the lipoxygenase and epoxygenase pathways (Smith and Murphy, 2002; Rahman et al, 1987; Dunn, 1983). These 20-carbon polyunsaturated fatty acids include: AA, also known as eicosa-5,8,11,14-tetraenoic acid; dihomo-γ-linolenic acid, also known as eicosa-8,11,14-trienoic acid; and EPA, also known as eicosa-5,8,11,14,17-pentaenoic acid. The prefix "eicos" is derived from the Greek word denoting "20", hence products derived from these compounds have been defined as eicosanoids. As reviewed by Harris in 2000, eicosanoids are produced by many tissues in the body, including brain, gut and kidney, and play various roles as local mediators of inflammation and as modulators of physiologic functions, such as maintenance of gastric mucosal integrity and modulation of renal microvascular hemodynamics, renin release, and tubular salt and water reabsorption. More specifically, PGI_2 is known to be a potent vasodilator and platelet

anti-aggregator, TXA_2 a potent vasoconstrictor and platelet aggregator and PGE_2, the predominate AA metabolite from the kidney, an antagonizer of vasopression, a hormone that increases water retention by the kidney (Fitzgerald et al, 1987; Needleman et al, 1986). Eicosanoids are not stored by cells. Instead, they are synthesized and rapidly released in response to extracellular hormonal stimuli where they act as local hormones as they are rapidly inactivated in the circulation (Smith and Murphy, 2002).

Products of the COX pathway give rise to PGs and TXs, collectively termed prostanoids. Products of the lipoxygenase pathway give rise to hydroperoxy eicosatetraenoic acids (HPETEs), hydroxy eicosatetraenoic acids (HETEs) and leukotrienes (LT). Products of the epoxygenase pathway give rise to epoxy eicosatrienoic acids (EETs). Each pathway is named after the enzyme that catalyzes the first step in the pathway. Please refer to Figure 1 for a more detailed description of the production of prostanoids by the COX pathway.

The COX enzyme, also known as PGH synthase, is the enzyme that catalyzes the first steps in the production of double bonded PGs and TXs from AA. Products of this pathway are denoted by a "2" subscript, which represents the double bond. PGs and TXs of this 2 series are the most common prostanoids formed (Smith and Murphy, 2002). Prostanoids containing one double bond are formed in the COX pathway from dihomo-γ-linolenic acid and therefore, form the "1" series of eicosanoids. Prostanoids containing three double bonds are formed in the COX pathyway from EPA and therefore, form the "3" series of eicosanoids. To denote the orientation of ring hydroxyl groups, Greek subscripts are used.

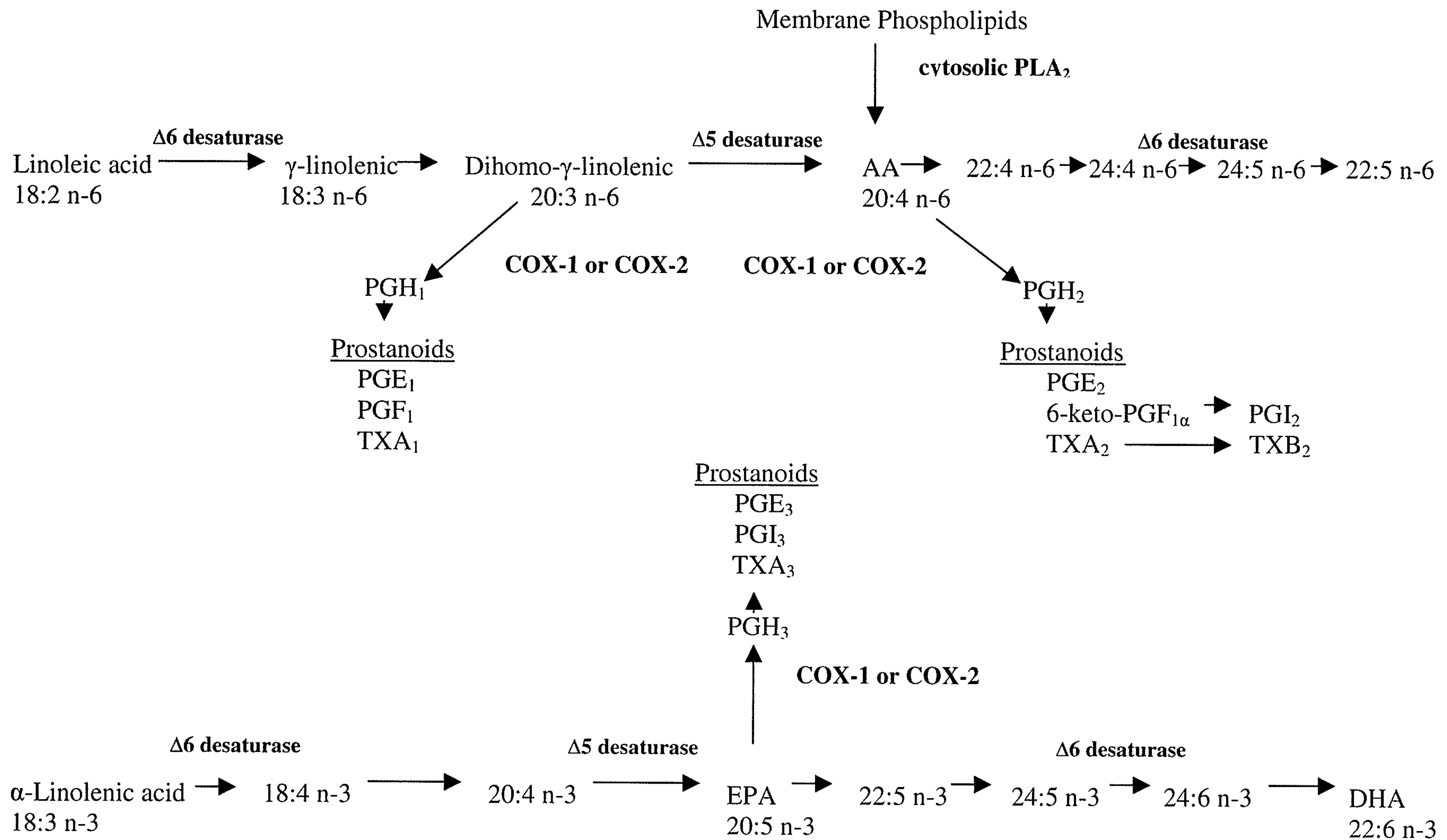

Figure 1 Production of prostanoids (adapted from Ferdinandusse et al, 2001; Sprecher, 2000; Crawford, 1983; and Horrobin, 1992)

In general, the enzyme systems for the metabolism of fatty acids are shared and therefore, competitive (Crawford, 1983). EPA and AA compete for entry in the COX and lipoxygenase pathway, however, AA is a better substrate for the COX pathway while EPA is a better substrate for the lipoxygenase pathway (Gibson, 1992; Willis, 1981). The precursors to eicosanoid formation can be derived from two sources, the metabolic pool and membrane phospholipids, which in turn can be affected by dietary lipid intake (Clark et al, 1990; Garg et al, 1988; Crawford, 1983). Alpha-linolenic acid, an n-3 fatty acid rich in flax oil can be desaturated and elongated to form EPA. Linoleic acid, an n-6 fatty acid rich in safflowerr oil, can be desaturated and elongated to form dihomo-γ-linoleic acid, which can be converted to AA. The Δ6 desaturase enzyme involved in the desaturation process is a rate-limiting step in the synthesis of the above mentioned COX pathway precursors (Crawford, 1983). Alpha-linolenic acid and linoleic acid are essential fatty acids for vertebrates because they lack the Δ12 and Δ15 desaturase enzymes which incorporate double bonds at these positions (Groff et al, 1995). Fish oil is rich in EPA. Evening primose oil is rich in γ-linolenic acid (pre-cursor to dihomo-γ-linolenic). Meats are a rich dietary source of AA.

In response to the hypothesis raised at the beginning of this section, Clark et al published a study in 1990 that provided support to the theory that n-6 and n-3 fatty acids can compete with one another to alter the lipid content of the kidney and thus the ratio of urinary PGI_2 to TXA_2 produced. The study involved 37 adult female Munich-Wistar rats that had undergone a 5/6 nephrectomy and were fed either high fish oil diet (24% wt/wt), high safflower oil diet (25% wt/wt), low protein diet (6% wt/wt) or control diet (regular lab chow) for one week prior to surgery followed by 28 days after surgery. Rats fed the

safflower diet had significantly higher AA levels in remnant kidney tissues compared to the fish oil fed and the control fed rats. Rats fed the fish oil diet had a significant reduction in AA in association with a rise in EPA and DHA. Urinary TXB_2 levels rose significantly in the rats fed safflower and control diets, however, rats fed fish oil showed only a slight rise in urinary TXB_2. At day zero, rats fed the fish oil and safflower oil diet for one week prior to surgery had significantly higher 6-keto-$PGF_{1\alpha}$ levels than the control rats. At day 28 the fish oil fed rats had a large significant decrease in urinary 6-keto-$PGF_{1\alpha}$ levels compared to day zero. Control fed rats showed a smaller significant decline, while safflower fed rats showed only a slight decline. The ratio of 6-keto-$PGF_{1\alpha}$ to TXB_2 declined in all experimental groups, however, the ratio was much greater for rats fed fish oil compared to safflower oil. Control rats had the smallest ratio. There was a significant positive correlation of renal tissue AA with increased urinary TXB_2. Also, fish oil fed rats had significantly reduced albuminuria. The researchers concluded that since the prostanoid assay used did not differentiate between prostanoids of the two and three series the competition of AA with EPA would not be expected to alter the prostanoids measured. Therefore, the prevention of the rise in TXB_2 by the fish oil diet is likely due to displacement of AA from the remnant renal tissue as supported by the lipid data, which in turn, may support the reduction in albuminuria.

Before PGs and TX of the 2 series can be formed AA must be released from the sn-2 position of membrane phospholipids. Although there may be a number of lipases and phospholipases that could remove AA from this position, PLA_2 appears to be the most important (Smith and Murphy 2002; Wilton and Waite 2002). Among the many PLA_2s, $cPLA_2$ is a ubiquitously distributed enzyme that is activated by an increase in

cytosolic Ca^{2+} and has a preference for AA at the sn-2 position of phospholipids (Wilton and Waite 2002; Evans et al, 2001; Hirabayashi et al, 2000; Bonventre, 1999; Hirabayashi et al, 1999; Gronich et al, 1988). The activated $cPLA_2$ translocates from the cytosol to the membrane surface, therefore, inactive $cPLA_2$ will be found in the cytosol fraction of the kidney homogenate while activated $cPLA_2$ will be found in the particulate fraction. After AA has been released the first step involves the conversion to PGH_2, by a COX enzyme. PGH_2 can then be transformed by a variety of enzymatic and non-enzymatic mechanisms into the primary prostanoids PGE_2, $PGF\alpha_2$, PGD_2, PGI_2 and TXA_2 (Vane et al, 1998).

As reviewed by Vane et al (1998), because of COX's role in the inflammatory process, pharmaceutical companies have developed drugs to target the inhibition of this enzyme in order to help alleviate the inflammatory process in a myriad of diseases, such as rheumatoid arthritis or osteoarthritis. These drugs include non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin. However, as reviewed by Harris in 2000, it was soon recognized that a second COX isoform may exist since studies involving cultured cells and tissues had shown that COX activity increased in response to mitogens (epidermal growth factor and human platelet-derived growth factor) and cytokines (interleukin-1) but this increased activity was due to the synthesis of a new protein (Harris and Badr, 1990; Raz et al, 1988; Habenicht et al, 1985). This second isoform (COX-2) was later described and found to be the main enzyme involved in inflammation (Kujubu et al 1991; Xie et al 1991). This new enzyme could be inhibited without inducing gastro-intestinal side effects observed from dual inhibition of COX-1 and COX-2.

There are now two known isoforms of COXs, COX-1 and COX-2, with speculation of a third (Willougby et al, 2000). Both COX-1 and COX-2 are inducible; COX-1 being present in nearly all cell types, while COX-2 is highly expressed in many tissues in response to inflammation (Aukema et al, 2002; Harris, 2000). COX-2 also seems to play an important role in the developing kidney (Langenbach et al, 1999). COX-2 expression in the macula densa/cortical thick ascending loop of Henle and medullary interstitial cells have been confirmed in the kidney of mice, rats, rabbits and dogs, as reviewed by Harris in 2000. Initial studies on COX-2 localization in the human kidney reported expression in podocytes and arteriolar smooth muscle cells and it was later shown to be expressed in the macula densa and medullary interstitial cells (Komhoff et al, 1999; Nantel et al, 1999; Komhoff et al, 1997).

The macula densa is involved in regulating renin release, thus implying a potential role of COX-2 in the renin-angiotensin-aldosterone system. Several studies have confirmed that COX-2 expression in the macula densa/thick ascending loop of Henle is increased in the presence of salt deficient diets and that when COX-2 is selectively inhibited in the presence of a salt depletion diet, the renal renin response is inhibited (Rodrígeuz et al, 2000; Yang et al, 1998; Harris et al, 1994). As alluded to above, the unfavourable increase in eicosanoid production seen in bilateral ureteral obstructed rats fed a high protein diet may be due to increased COX enzyme activation brought about by angiotensin II, which is involved in the renin-angiotensin-aldosterone system. (Yanagisawa, 1994; Yanagisawa et al, 1991; Yanagisawa et al, 1990).

The renin-angiotensin-aldosterone system stimulates sodium reabsorption in the distal and collecting tubules of the nephron. The granular cells of the juxtaglomerular

apparatus, which also contains the tubular macula densa cells, secretes renin in response to decreased plasma sodium, extracellular fluid volume or blood pressure. Renin first converts angiotensinogen (synthesized by the liver and always present in plasma in high concentrations) to angiotensin I. Angiotensin I passes through the lungs where it is converted to angiotensin II by the angiotensin converting enzyme. Angiotensin II is the primary stimulus for the secretion of aldosterone from the adrenal gland. Aldosterone is then responsible for the reabsorption of sodium by the distal and collecting tubules of the nephron by stimulating the synthesis of new proteins within the tubular cells. The synthesis of these proteins allows for sodium to be reabsorbed either passively through the formation of sodium channels in the luminal membranes or actively through the formation of Sodium-Potassium-ATPase carriers, which are inserted into the basal lateral membranes of the cells. Aldosterone itself is able to aid in increasing blood pressure by the constriction of arterioles, stimulation of thirst and stimulation of vasopressin.

In 1993, Lewis et al, confirmed the beneficial effects of angiotensin-converting-enzyme inhibitors, such as captopril on the progression of DN in Type 1 diabetes patients, independent of blood pressure control (systolic, diastolic and arterial), as seen by a 48% reduction in the risk of doubling serum creatinine and a significant reduction in proteinuria. In September 2001, two articles were published in the New England Journal of Medicine which investigated the role of angiotensin II-receptor antagonist in patients with Type 2 diabetes who also had MA or DN. Parving et al (2001) studied the effect of irbesartan in hypertensive patients with Type 2 diabetes and MA and concluded that this angiotensin II-receptor antagonist significantly reduces the rate of progression to DN independent of its blood-pressure-lowering effects. Brenner et al (2001) found similar

results when they studied the effects of losartan in patients with Type 2 diabetes and DN. They concluded that this angiotensin II-receptor antagonist reduced the risk of ESRD by 28% which corresponds to an average delay of 2 years in the need for dialysis or transplant.

In 2001, Komers et al studied the role of renal COX-1 and COX-2 protein expression and renal and systemic hemodynamic responses to inhibition of each enzyme with valeryl salicylate and NS398, respectively, in T1 rats. COX-2 protein expression was found to be significantly higher in the renal cortex of rats with poor glycemic control compared to those with good glycemic control and control rats. This increase was normalized with improved metabolic control. COX-1 protein expression was not different among the experimental groups. COX-2 inhibition reduced GFR in T1 rats as well as urinary flow. This was accompanied by a stable renal plasma flow and renal vascular resistance. Plasma renin concentration was significantly decreased in T1 rats treated with the COX-2 inhibitor compared to the control rats treated with COX-2 inhibitor. T1 rats had higher urinary PGE_2 and TXB_2 than control rats at baseline; however, TXB_2 levels were higher only after data for the T1 rats were pooled. Both inhibitors significantly decreased urinary excretion of PGE_2 versus baseline. Only the COX-2 inhibitor significantly reduced the excretion of TXB_2 versus baseline. Both inhibitors had no effect on mean arterial pressure. The researchers concluded the there is a role for COX-2 derived PG in the pathogenesis of hemodynamic changes observed in DN. Possibly mechanisms of action described include: 1) activation of protein kinase C by hyperglycemia, which in turn has been shown to activate PLA_2 and therefore the release of AA with subsequent PGE_2 production, 2) activation of COX-2 by

glycosylation products, 3) enhanced COX-2 expression/activation by angiotensin II. In 2002, Komers et al further studied the effects of COX-2 inhibition on plasma and renal renin in T1 rats and concluded that COX-2 does not play a significant role in mediating renin status in this animal model of Type 1 diabetes.

PGE_2 and PGI_2 are known to have well defined renal functions (Harris, 2002; Brater et al, 2001). PGE_2 acts as a counter-regulatory factor in the regulation of sodium resorption under conditions where sodium resorption is increased. PGI_2 is able to stimulate the secretion of renin and activate the renin-angiotensin-aldosterone system which in turn increases potassium secretion. In addition, because of PGI_2s vasodilatory properties, it will increase renal blood flow and GFR under conditions associated with decreased circulating volume, thus resulting in greater renal tubular flow and the secretion of potassium. As a consequence, inhibition of the COX enzymes, and thus the production of PGs can ultimately result in increased sodium resorption, and therefore, edema as well as decreased GFR and increased potassium in the blood. PGs do not play a major role in sodium and water homeostasis in healthy hydrated individuals (Whelton, 2000).

Analogs of TXA_2 and PGI_2 alter the expression of genes encoding basement membrane proteins *in vitro* in differentiated mouse tetrocarcinoma cells and human glomerular mesangial cells (Bruggeman et al, 1993; Bruggeman et al, 1991). TXA_2 analogs increase the steady-state mRNA levels for all three laminin chains (A, B1, B2), type IV collagen and fibronectin and decrease the level of mRNA for heparin sulfate proteoglycan, while PGI_2 has the opposite effect. As stated above, thickening of glomerular and tubular basement membranes is characteristic of DN progression. In

1990 Ledbetter et al concluded that basement membrane thickening in a Type 2 diabetes mouse model is partly a result of an unbalanced increase in the production of type IV collagen and that inhibition of TXA_2 prevented an increase in type IV collagen mRNA.

Further investigation into which dietary fats can affect renal disease progression via alteration of eicosanoid production presents an interesting area of research. More specifically, the emerging benefical health effects of dietary CLA makes the investigation of this specific type of fat, in relation to eicosanoid production and renal disease, a fascinating area of research.

2.4.1 CLA and Eicosanoids

Conjugated linoliec acid (CLA) is the generic name given to a mixture of positional (e.g. 8, 10; 9,11; 10,12; 11,13) and geometric (*cis* or *trans*) isomers of linoleic acid (Moya-Camarena and Belury, 1999; Parodi, 1997). CLA is formed when one or both of the double bonds of linoleic acid are moved such that double bonds are no longer separated by two single bonds. Several CLA isomers have been identified in food, but the *cis*-9, *trans*-11 configuration is the most common (Kelly, 2001; Pariza et al, 2000).

CLA is formed in the rumen of animals, such as cows. Milk fat is the richest dietary source of *cis*-9, *trans*-11 (Parodi, 1997). CLA is formed in ruminant animals as a first intermediate in the biohydrogenation of dietary linoleic acid by a linoleic acid isomerase enzyme from the rumen bacteria *Butyrivibrio fibrisolvens* (Parodi, 1997). The formation of CLA from linoleic acid can also occur under strong alkaline conditions during commercial hydrogenation of vegetable oils and at high temperatures (Öhman et al, 2002). To date, the majority of research has been conducted using mixtures of CLA

isomers (Kelly, 2001). The *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 CLA isomers make-up approximately 85-90% of these mixtures in about equal amounts with the rest of the mixture being composed of approximately 10 other CLA isomers. Mixtures are usually about 90% pure CLA (Belury, 2002; Kelly, 2001; Pariza, 2000).

As reviewed by Belury in 2002, CLA has been shown to have several beneficial health consequences, some of which include anti-adipogenic, anti-diabetogenic, anti-carcionogenic and anti-atherosclerotic effects. The mechanisms involved are hypothesized to involve the modulation of eicosanoid production, enhanced oxidation of lipids, cell apoptosis and/or differentiation, decreased cell proliferation and alterations in gene expression.

Diets rich in CLA are able to increase the level of CLA in human and animal tissues, but it is generally low and varies depending on the tissue and CLA isomer (Alasnier et al, 2002; Belury, 2002; Banni et al, 2001; Jiang et al, 1999; Li and Watkins, 1998; Parodi et al, 1997). In addition, isomers of CLA can be elongated and desaturated to form conjugated forms of linoleic acid metabolites, including conjugated-AA, which may result in a reduced AA pool and reduced production of AA derived eicosanoids (Belury, 2002; Sébédio et al, 1999; Sébédio et al, 1997; Banni et al, 1996). CLA mixtures have been shown to reduce PGE_2 in serum and *ex vivo* bone organ culture of Sprague-Dawley rats as well as cell culture macropahges, but not in the spleen of Sprague-Dawley rats (Yu et al, 2002; Li and Watkins, 1998; Sugano et al, 1998). In addition, CLA mixtures inhibit the formation of TXA_2 in cultured human platelets, while various isomers increase or decrease PGI_2 formation in cultured human endothelial cells and platelets, depending on whether or not CLA is in the free fatty acid form or esterified

into cellular lipids and whether cells are in the resting or stimulated state (Torres-Duarte et al, 2003; Truitt et al, 1999). Recently, it has been confirmed that dietary CLA competes with linoleic acid in a Polycystic Kidney Disease rat model and inhibits the production of PGE_2 (Ogborn et al, 2003 – in press). In addition, recent unpublished data from Dr. Aukema's lab has revealed that in this model, CLA alters protein levels of COX-1, COX-2 and $cPLA_2$. In 2002, Belury discussed three possible theories for how CLA may reduce AA-derived eicosanoid products. These theories include:

1. CLA may displace AA in phospholipids,
2. CLA may inhibit COX-1 and/or COX-2 enzymes at the level of mRNA, protein or activity,
3. CLA or its elongated and desaturated products may act as substrates or antagonists for COX-1 and/or COX-2, therefore reducing enzymes available for AA.

Research in elucidating the mechanisms involved in CLA action is a relatively new area and it poses many challenges due to the fact that different CLA isomers may have different mechanisms of action in different tissues, as discussed above.

In 1998, Houseknecht et al provided the first evidence that a CLA mixture possessed anti-diabetogenic properties in male Zucker diabetic fatty (ZDF) rats, an animal model of Type 2 diabetes mellitus. The experimental diets consisted of 1.5% (wt/wt) CLA, no CLA or 0.2% troglitazone, a synthetic insulin sensitizing agent of the TZD family. Diets were isocaloric and were formulated according to a modified AIN-76 mixture containing 6.5% fat by weight. A CLA dose of 1.5% (wt/wt) was chosen based on previous evidence that showed beneficial effects of dietary CLA at this level (Belury

et al, 1997; Belury et al, 1996). At six weeks of age, ZDF rats and lean littermates were assigned to receive one of the three experimental diets for two weeks after which time they were terminated. CLA normalized glucose tolerance, improved hyperinsulinemia and lowered circulating free fatty acids, therefore, preventing or delaying hyperglycemia in the ZDF rats, similar to troglitazone treated rats. In addition, ZDF rats fed CLA and troglitazone had significantly increased fat cell differentiation compared to controls, after treatment for 14 days. The ZDF rats fed CLA had significantly lower final body weights than troglitazone fed ZDF rats.

A follow-up study was conducted to elucidate the anti-diabetogenic effects of the specific CLA isomers in the ZDF rats, which was the first of its kind in diabetes research (Ryder et al, 2001). Experimental diets included control, 1.5% CLA containing a 50:50 mixture of *cis*-9, *trans*-11 CLA and *cis*-10, *trans*-12 CLA or 91% *cis*-9, *trans*-11 CLA from butterfat. The 50:50 diet reduced adiposity, improved glucose tolerance and improved insulin-stimulated glucose transport and glycogen synthase activity in skeletal muscle compared to those fed the control diet and those fed the 91% *cis*-9, *trans*-11 CLA diet. Even though the 50:50 fed rats and pair-fed rats ate less food and lost weight, the 50:50 fed rats still showed better glucose tolerance, better insulin-stimulated glucose transport and glycogen synthase activity in muscle compared to the pair-fed rats, suggesting that the effects of the 50:50 diet could not be explained by reduced food intake alone. Results also indicate that the *cis*-9, *trans*-11 CLA isomer is not the primary isomer involved in CLA's anti-diabetogenic effects and that the *cis*-10, *trans*-12 CLA isomer plays a larger role. In 2003, Belury et al found that the *trans*-10, *cis*-12 CLA

isomer and not the *cis*-9, *trans*-11 CLA isomer was inversely associated with changes in body weight and serum leptin levels in patients with Type 2 diabetes.

The anti-diabetic properties of CLA in the Houseknecht et al (1998) and Ryder et al (2001) studies discussed above appear to be linked to CLA-activation of PPARs.

2.5 PPARs

There are four known PPAR isoforms, which include α, β/δ or NUC1, and γ, where PPAR γ exists as two isoforms, γ_1 and γ_2 (Asano et al, 2000). PPARs received their name from the fact that upon their discovery in 1990 they were able to aid in the proliferation of peroxisomes after they had been activated by peroxisome proliferators, such as fibrates (Issemann and Green, 1990). Peroxisome proliferation is an event that is limited to the liver and kidney tissues (Schoonjans et al, 1996). Peroxisomes are membrane-bound sacs that contain oxidative enzymes and the catalase enzyme. Peroxisomes use their oxidative enzymes to detoxify the cell of harmful waste. The major product generated in this process is hydrogen peroxide, which can also be harmful to the cell. However, the catalase enzyme contained within the peroxisome decomposes the hydrogen peroxide to harmless oxygen and water (Sherwood, 1993). Oxidative enzymes found in peroxisomes are associated with the β-oxidation of very long-chain fatty acids as well as PGs and LTs (Keller et al, 2000). Peroxisome enzymes are also involved in fatty acid elongation, the hydrolysis of acyl-CoAs and the conversion of acyl-CoAs to acylcarnitines, the biosynthesis of cholesterol and the metabolism of glucose via the hexose monophosphate shunt, to name a few (Schoonjans et al, 1996). PPARs, however, not only aid in the proliferation of peroxisomes but also enhance or

suppress the transcription of various target genes. PPARs play important roles in the general transcription of several cellular processes, including lipid metabolism, glucose homeostasis, cell cycle progression, cell differentiation and inflammation (Houseknecht et al, 2002; Picard and Auwerz, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Gregoiré et al, 1998; Schoonjans et al, 1996).

More specifically, PPAR α regulates the expression of genes involved in lipid transport and lipid oxidation, mainly in the liver and oxidative muscles, as well as anti-inflammation (unpublished data Dr. Taylor; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Devchand et al, 1996; Schoonjans et al, 1996). PPAR γ is a key transcription factor involved in terminal differentiation of adipocytes and also regulates genes involved in anti-inflammation and glucose homeostasis (Houseknecht et al, 2002; Picard and Auwerz, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Gregoire et al, 1998; Schoonjans et al, 1996). The specific function of PPAR β is yet to be elucidated. It has been suggested as having an important role in cell survival and mouse embryonic development (Keller et al, 2000; Wu, 2000). In addition, PPAR β agonists have been shown to have favorable effects on blood lipid profiles in insulin resistant db/db mice as reflected by increased high density lipoproteins (Leibowitz et al, 2000).

PPAR molecules are organized into structural and functional domains (Houseknecht et al, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Schoonjans et al, 1996). The NH2-terminal A/B domain contains a constitutive ligand-independent transactivation function and phosphorylation of the domain modulates the receptor activity. The C domain contains the DNA-binding domain which

targets the PPAR receptor to specific DNA sequences on a gene, known as peroxisome proliferators response elements (PPREs). The D domain serves as a hinge allowing the PPAR protein to interact with co-activators or repressors. The large E/F domain has several functions. In addition to ligand binding, it is required for nuclear localization, heterodimerization and ligand-dependent transactivation.

PPARs regulate gene transcription by first binding to a ligand. The ligand-bound PPAR then forms a heterodimeric complex with 9-*cis*-retinoic acid receptor or RXR, which also is a transcription factor. This heterodimeric complex is responsible for the transcriptional properties of PPARs as this complex then binds to the PPRE on DNA. Accessory proteins known as co-activators or co-repressors can also bind to PPARs in a ligand dependent manner and affect the transcriptional process.

Ligands for PPARs or peroxisome proliferators include synthetic and natural forms (Corton and Anderson, 2000). Ligands for PPAR α include: synthetic fibrates, natural monounsaturated fatty aids, such as oleic acid, polyunsaturated fatty acids, such as CLA, linoleic acid, α-linolenic acid, γ-linolenic acid, AA, EPA, and DHA as well as the PGs PGA_1, PGD_2, PGJ_2 and LTs, such as HETEs (Fruchart et al, 1999; Moya-Camarena et al, 1999; Ouali et al, 1998; Forman et al, 1997; Kliewer et al, 1997; Yu et al, 1995). Ligands for PPAR γ include: synthetic TZDs, natural monounsaturated fatty aids, such as oleic acid, polyunsaturated fatty acids, such as CLA, linoleic acid, α-linolenic acid, γ-linolenic acid, AA, EPA, and DHA as well as the PGs PGA_1, PGD_2, PGJ_2 and LTs, such as HETEs (Houseknecht et al, 1998; Forman et al, 1997; Kliewer et al, 1997; Berger et al, 1996; Willson et al, 1996; Lehmann et al, 1995; Yu et al, 1995). Ligands for PPAR β include synthetic fibrates and TZDs as well as natural linoleic acid, α-linolenic

acid, γ-linolenic acid, AA, EPA, DHA, PGJ_2 and HETE (Leibowitz et al, 2000; Forman et al, 1997).

Northern hybridization and RNase protection assays show that all three PPAR isoforms can be found in the kidney as well as the ureter and bladder (Guan et al, 2001; Guan and Breyer, 2001; Nicholas et al, 2001; Asano et al, 2000; Yang et al, 1999; Guan 1997; Braissant 1996). PPAR α is most abundantly expressed in proximal tubules and medullary thick ascending limbs of both humans and rabbits and the proximal convoluted tubules of the rat. PPAR γ is selectively expressed in inner medullary collecting ducts and pelvic urothelium and glomerular and mesangial cells of the kidney of humans and rabbits and in the inner medullary collecting duct, medullary interstitial cells and mesangial cells of rats. PPAR β is ubiquitously expressed in all segments of the nephron as well as the renal cortex and medulla of humans and rabbits as well all segments of the nephron in rats.

As stated above, TZDs are ligands for PPARs, particularly PPAR γ (Guan et al, 2001; Berger et al, 1996; Willson et al, 1996; Lehmann et al, 1995). TZDs are used as insulin sensitizing agents in some Type 2 diabetes patients and their effect is thought to be modulated by the activation of PPAR γ (Picard and Auwerx, 2002). As poor glycemic control is a risk factor for DN, it would be thought that better control of blood glucose levels obtained by TZD users would result in renal protective effects. Indeed, TZDs have been shown to ameliorate MA in the TI rat and Type 2 diabetes patients (Imano et al, 1998; Fujii et al, 1997). In studies involving the obese *fa/fa* Zucker rat, a model of obesity and the Type 2 diabetes "pre-diabetic" state, and the ZDF rat, a model of Type 2 diabetes, TZDs decreased proteinuria and halted the early onset and progression of

mesangial expansion and glomerulosclerosis (McCarthy et al, 2000; Buckingham et al, 1998). It has also been shown in the T1 rat that TZDs are able to reduce mRNA expression of the extra cellular matrix proteins fibronection and type IV collagen, without changing blood glucose levels (Isshiki et al, 2000). The fact that TZD-treated T1 rats, which produce no insulin, had ameliorated MA and reduced mRNA levels of extra cellular matrix proteins supports the possibility that TZDs may have direct beneficial renal effects independent of their capability to improve glucose tolerance.

Recent studies involving rabbits and T1 rats have shown that PPAR γ ligands, such as TZDs can significantly inhibit proliferation of mesangial cells in a dose dependent manner (Guan et al, 2001; Nicholas et al 2001; Asano et al, 2000). In addition, it has been shown that patients with Type 2 diabetes who carry a PPAR-γ2 Ala12 allele have significantly lower proteinuria and develop overt proteinuria less frequently (Herrmann et al, 2002). This implies that PPAR γ activation may reverse the phenotypic change of mesangial cells and inhibit cell growth and reduction of extra cellular matrix proteins seen in DN.

2.5.1 CLA and PPARs

As stated above, CLA is a ligand for PPAR α and PPAR γ (Moya-Camarena et al, 1999; Houseknecht et al, 1998). Referring back to the Houseknecht et al (1998) study, which showed that CLA acted similarly to TZD in that it normalized glucose tolerance, improved hyperinsulinemia and lowered circulating free fatty acids in ZDF rats, it was also found that increasing levels of CLA induced a dose-dependent transactivation of PPAR γ in African green monkey kidney cells, which was consistent with a significant

increase in fat cell differentiation seen in the CLA fed and TZD fed rats. In the Ryder et al (2001) study, there was no effect of CLA treatment on PPAR γ expression in muscle or adipose tissue even though their results are consistent with PPAR γ activation. Results from both studies also suggest CLA activation of PPAR α since circulating free fatty acids were reduced, although this was not measured. In 1999, Moya-Camarena et al found that CLA is a potent naturally occurring ligand and activator of PPAR α in rat hepatoma cells. In addition, it has recently been reported that CLA is able to increase mRNA levels of PPAR α responsive genes in the liver of obese *fa/fa* Zucker rats (Yurkova et al, 2002).

As hyperlipidemia is a risk factor for DN, it would be thought that better control of blood lipid levels with the use of fibrates would result in renal protective effects. One of the main characteristic of the obese *fa/fa* Zucker rat is hyperlipidemia and the development of glomerularsclerosis by 28 weeks of age (Upton et al, 1998; Kasiske et al, 1985). Reduction of hyperlipidemia in the obese *fa/fa* Zucker rat after approximately 32 weeks of fibrate treatment did significantly reduce proteinuria, mesangial expansion and glomerularsclerosis as reported by Kasiske et al, 1988. Because fibrates are PPAR α ligands, PPAR α may have a beneficial role to play in attenuating DN.

Therefore, investigation into the possible benefical effects of dietary CLA on renal disease progression, as it relates to eicosanoid production and PPAR levels, in the obese *fa/fa* Zucker rat would provide useful insight into the possible deliourious mechanism underlying early DN.

2.6 The Obese *fa/fa* Zucker Rat

The obese *fa/fa* Zucker rat is a model of obesity that results from an autosomal recessive trait (Bray, 1977, Zucker, 1965). A *fa* mutation in the leptin receptor interferes with leptin's ability to suppress appetite and mediate theromgenic actions, therefore resulting in hyperphagia, obesity, hyperlipidemia, hyperinsulinemia, peripheral insulin resistance and impaired glucose tolerance (Phillips et al, 1999; Upton et al, 1998; Kasiske et al, 1992; McCaleb and Sredy, 1992; Ionescu et al, 1985). As a consequence, the male obese *fa/fa* Zucker rat resembles human obesity and impaired glucose tolerance, which can eventually lead to Type 2 diabetes mellitus and renal complications associated with this disease.

Renal impairment has been observed in the obese *fa/fa* Zucker rat. In 1985, Kasiske et al found that obese male *fa/fa* Zucker rats developed glomerular mesangial matrix expansion by 14 weeks of age, which was followed by a rapid increase in albuminuria. Inulin clearance and filtration fraction remained normal while these changes occurred, suggesting increased GFR and renal plasma flow is not a prerequisite for focal glomerular sclerosis. By 28 weeks of age, focal glomerular sclerosis was evident and by 68 weeks it was extensive. Increased mean systolic blood pressure was observed in all rats throughout the study. It was hypothesized that hemodynamic and metabolic factors are involved in the pathogenesis of this non-immune mediated renal disease and in 1985 O'Donnell et al published a study that investigated whether specific changes in glomerular hemodynamics played a role. The researchers concluded that metabolic abnormalities are more likely as hemodynamic alterations did not appear to initiate glomerular injury.

In 2000, Coimbra et al assessed age-related renal changes in obese *fa/fa* Zucker rats. Six male obese *fa/fa* Zucker rats were studied at 6, 10, 14, 18, 40, and 60 weeks of age, while 6 male lean Zucker rats ("controls") were observed at 6, 14, and 40 weeks of age. The three early pathological events that were considered significant were: 1) podocyte injury, 2) increased glomerular monocyte/macrophage counts, and 3) glomerular hypertrophy. Similar findings were reported in a review by Phillips et al in 1999. At 6 weeks of age, there was significantly increased *de novo* expression of desmin in individual podocytes, which progressively increased until 18 weeks. Podocytes only express desmin following various types of renal injury, such as glomerular hypertension and immune-mediated or toxic injury. At 10 weeks of age, the first evidence of podocyte activation and damage was noted as depicted by an increase in the number of mitochondria. After 10 weeks of age, progressive glomerular hypertrophy was detected. At points past 10 weeks of age type IV collagen and fibronectin showed mild increases in the glomerular and/or tubulointerstitial compartments of the extra cellular matrix. Mesangial cell proliferation and activation showed only a low-grade increase as depicted by glomerular α-smooth muscle actin expression. At 14 weeks of age, glomerular and tubulointerstitium monocyte/macrophage infiltration was at its peak. Other relevant finding included significantly increased GBM width and tubular cell damage at 14 weeks of age.

3 METHODS AND MATERIALS

3.1 Experimental Design

Male obese *fa/fa* Zucker rat kidneys for this study were obtained from a study conducted by Dr. Taylor in the summer of 2001. Male obese *fa/fa* Zucker rats were used in this study as a model for human obesity and impaired glucose tolerance or the Type 2 diabetes "pre-diabetic" state. As a consequence, early dietary intervention and its effect on disease progression can be evaluated. In addition, in terms of PPAR function, male Sprague-Dawley, lean and obese ZDF and Fisher 344 rats fed CLA have been shown to have greater induction of PPAR-responsive genes in the liver compared to female rats (Moya-Camarena and Belury, 1999). The rats were purchased from Charles River, St. Constant, Quebec. The study involved 20 obese *fa/fa* Zucker rats: 10 were fed a diet containing 1.5% CLA (faCLA) and the other 10 were fed a diet containing 0% CLA (faCTRL). It also involved 20 lean Zucker rats: 10 were fed a diet containing 1.5% CLA (lnCLA) and the other 10 were fed a diet containing 0% CLA (lnCTRL). The percentage dietary CLA refers to the grams of CLA oil per kilogram of diet. The amount of CLA used in the experiment (i.e. 1.5%) was chosen based on the Houseknecht et al (1998) study discussed above and was purchased from NuChek Prep, Elysian, Minnesota. Please refer to Table 3 for a complete list of diet ingredients and Table 4 for composition of CLA mixture. The CLA mixture was 86.3% pure and was in the free fatty acid form. It was composed of four major isomers: *trans*-10, *cis*-12 CLA; *cis*-9, *trans*-11 CLA; *cis*-11, *trans*-13 CLA; and *trans*-8, *cis*-10 CLA (in order from most to least abundant). The dry ingredients for the diets were pre-mixed and fresh batches of feed containing oil (CLA = 1.5% + 7% soy oil; CTRL = 8.5% soy oil) were prepared weekly and stored at

Table 3 Composition of CLA and CTRL diets. Diets were based on the AIN-93G diet for laboratory rodents. [+]

Ingredient (g/kg of diet)	CLA	CTRL
Cornstarch [a]	347.6	347.6
Maltodextrin	132	132
Sucrose	100	100
Egg White	212.5	212.5
Fibre (cellulose)	50	50
Mineral mix (zinc-free) - AIN-93G	35	35
Vitamin Mix - AIN-93G	10	10
*Zinc premix [c]	10	10
**Biotin Premix	10	10
Potassium phosphate [b]	5.4	5.4
Choline	2.5	2.5
Tert-butylhdroquinone	0.014	0.014
***Soybean Oil [d]	70	85
****CLA [e]	15	0

*Zinc premix = 5.775 g Zn carbonate + 994.2 g cornstarch
**Biotin premix = 200 mg biotin + 1 kg cornstarch
***Soybean oil given as 7% by weight for CLA diet and 8.5% by weight for CTRL diet.
****CLA given as 1.5% by weight for CLA diet and 0% by weight for CTRL diet.
All ingredients without a superscript were supplied by Harlan Teklad, Madison, Wisconsin.
[a] supplied by the University of Manitoba, Winnipeg, Manitoba
[b] supplied by Fisher Scientific
[c] zinc carbonate (in Zinc premix) supplied by Fisher Scientific
[d] supplied by Vita Health, Winnipeg, Manitoba
[e] supplied by NuCheck Prep, Elysian, Minnesota
[+] Reeves et al, 1993.

Table 4 Composition of CLA mixture.

CLA Isomer	% of total CLA
? CLA	0.08
? CLA	0.05
9c11t	28.63
8t10c	15.54
10c12t	0.79
11c13t	17.75
10t12c	29.89
8c10c	0.87
9c11c	1.55
10c12c	1.53
11c13c	0.74
? CLA	0.08
11t13t	0.23
9t11t/10t12t	2.26
Total cis, trans CLA	92.60
Total cis, cis CLA	4.69
Total trans, trans CLA	2.50
% CLA as Total Fat	86.30

Analysis of the CLA mixture was performed by Dr. Kramer of the Food Research Program at Agriculture and Agri-Food Canada.
CLA purchased from NuChek Prep, Elysian, Minnesota.
c = *cis*
t = *trans*

-20°C. Feed consumption (corrected for spillage) and weekly body weights were recorded. Rats were obtained at 5 weeks of age and were subject to a one-week acclimatization period. The feeding study lasted for 8 weeks in which the rats were given new feed cups with fresh feed three times per week. Animals were housed individually in hanging stainless steal wire-bottom cages. During the treatment period, rats were kept in a controlled environment in which the temperature was maintained at 21-23°C with 55% relative humidity and a 14 hr light, 10 hr dark cycle. At week 7, urine was collected during a 12 hr overnight fast while being housed in polycarbonate metabolic cages (Nalgene, Fisher Scientific, Fair Lawn, New Jersey). The urine samples were collected in pre-weighed vials and weighed to determine the amount of urine collected by subtraction. The urine was then frozen at -20°C for later analysis. At the end of 8 weeks, rats were fasted overnight for 12 hr and then asphyxiated with CO_2 before collection of blood and tissues. Kidneys were weighed and frozen immediately in liquid nitrogen and stored at -80°C except for the longitudinal cross-section of one kidney which was placed in 10% phosphate buffered formalin (Fisher Scientific, Fair Lawn, New Jersey) for histopathology.

3.2 Measurement of Mean Glomerular Volume

Longitudinal kidney cross-sections that were placed in 10% phosphate buffered formalin were processed into paraffin wax and sectioned at 5 microns using a Microtome (American Optical 820, Southbridge, Massachusetts). Sections were then placed in xylene to remove paraffin before being hematoxylin and eosin stained. Sections were stained in Harris's hemotoxylin for 5 minutes, washed in deionized water, differentiated

in 1% HCl in deionized water and then blued in 2% ammonia water. Hematoxylin stains negatively charged nuclei and ribosomes blue. Next the sections were washed with deionized water and placed in eosin for 3 minutes after which time they were dehydrated in alcohol and mounted with cytoseal. Eosin stains proteins pink.

Using the procedure developed by Wiebel (1979), MGV was determined by first measuring glomerular diameter of at least 30 randomly chosen glomeruli per kidney by light microscopy on an Olympus BX60 microscope using a 20X objective. The microscope was connected to a Junior Spot CCD high-resolution camera (Diagnostic Instruments Inc, Sterling Heights, Michigan) and the image taken was projected onto a Sony 19 inch monitor with the use of Spot Advanced software version 3.0.1 (Diagnostic Instruments Inc, Sterling Heights, Michigan). Using Image Pro Plus software version 4.10 (Media Cybernetics, Del Mar, California) and a 20X calibration grid, the diameter of each glomerulus was measured by point counting. Each diameter measurement (μm) was divided by two to obtain the radius of each glomerulus and the average radius (r) was calculated. Mean glomerular area (MGA) was calculated as:

$$MGA = \pi r^2$$

MGV was calculated as:

$$MGV = 1.25\,(MGA)^{3/2}$$

where, 1.25 is derived from β/K, where β equals 1.38 (pertains to spheres) and K equals 1.10 (a distribution coefficient) (Hirose et al, 1982). This method of assessing MGV has been published in studies involving the obese *fa/fa* Zucker rat (Maddox et al, 2002).

3.3 Measurements of Kidney Function

3.3.1 Urine Creatinine

Urine creatinine was measured using a creatinine kit (Sigma, catalogue # 555-A). Creatinine is synthesized from free creatine in muscle and the amount of creatinine produced is proportional to muscle mass. In the absence of renal disease, the excretion rate of creatinine is relatively constant. Therefore, measurement of urinary creatinine, serum creatinine and creatinine clearance are useful markers of kidney function.

The assay is based on the formation of a yellow/orange colour when creatinine reacts with alkaline picrate. However, because this reaction is not specific, as there are a number of proteins in body fluids that can interfere, an acid reagent is added such that the creatinine-picrate colour fades faster than the colour produced by interfering factors. The difference in colour intensity measured at 500 nm before acidification and after acidification is proportional to the creatinine concentration in the sample.

A concentration curve was generated to confirm the linearity range of the assay using dilutions of a 15 mg/dL creatinine standard to yield 3 mg/dL, 5 mg/dL, and 10 mg/dL creatinine standards. The concentration of creatinine in urine samples was calculated based on comparison to a 3 mg/dL creatinine control. Urine samples were diluted 20 fold for the test. One hundred μL of blanks (water), standard or samples, were added, in duplicate, to 1.5 mL semimicro polystyrene cuvettes (Fisher Scientific, Fair Lawn, New Jersey). One mL of alkaline picrate solution (5 volumes of picric acid with 1 volume of sodium hydroxide solution, provided by the kit) was added to each cuvette and incubated for 10 minutes at room temperature. The colour intensity produced in each cuvette was measured at 500 nm using a Milton Roy Spectronic 3000 Array

spectrophotometer (Fisher Scientific, Fair Lawn, New Jersey). Next, 33.3 μL of acid reagent (a mixture of sulfuric acid and acetic acid, supplied with the kit) was added to each well, mixed thoroughly and allowed to stand for 5 minutes at room temperature after which time a second absorbency reading was taken at 500 nm. Creatinine concentration of the unknown samples was then calculated using the following equations:

Creatinine Concentration (mg/dL) =

$$\left[\frac{\textbf{initial A of unknown sample} - \textbf{final A of unknown sample}}{\textbf{initial A of 3 mg/dL standard} - \textbf{final A of 3 mg/dL standard}}\right] \times \textbf{3 mg/dL}^{*} \times \textbf{Dilution Factor}$$

where, A = absorbance and * is the concentration of the creatinine standard.

The value of creatinine concentration was then multiplied by the dilution factor (20) before being converted into milligrams of creatinine in a 12 hr urine sample, which was calculated using the following equation:

Creatinine Excretion (mg/12 hr) =

Creatinine Concentration (mg/dL) X dL of urine collected in 12 hrs

3.3.2 Serum Creatinine

The same procedure was used to measure serum creatinine (mg/dL) as was used to measure urine creatinine (mg/dL). However, because the serum creatinine concentration of the unknown samples were low, the 15 mg/dL creatinine standard was diluted to 0.2 mg/dL, 0.5, mg/dL and 1 mg/dL to provide a similar concentration to the serum creatinine concentrations of the unknown samples. The serum creatinine concentrations were still detectable at the lower range of the standard curve and were within the linear range of the assay.

3.3.3 Creatinine Clearance

Creatinine clearance was calculated using the following equation:

Creatinine Clearance (mL/min) =

$$\frac{\textbf{urine creatinine (mg/dL) X urine volume (dL/12 hr)}}{\textbf{serum creatinine (mg/dL)}}$$

where, 12 hr was expressed in minutes.

3.3.4 Urine Protein

Urine protein was measured by first removing interfering substances, such as sucrose and salts, by precipitation with an ice cold 20% tricholoracetic acid (TCA) solution (Fisher Scientific, Fair Lawn, New Jersey). Fifty µL of the 20% TCA was added to 50 µL of urine sample while vortexing. Samples were centrifuged at 4 °C (IEC Micromax, International Equipment Company, Needham Heights, Massachusetts) for 5 minutes at 15,000 rotations per minute. The supernatant fraction was discarded and the remaining protein pellet was resuspended in 100 µL of phosphate buffered saline (PBS, Sigma, St. Louis, Missouri) by vortexing. Ten µL of resuspended protein was diluted with 40 µL of PBS to give a 1:4 ratio or 5-fold dilution of resuspended protein in PBS.

Ten µL of diluted urine protein samples were added, in triplicate, to a Costar 96-well (Conring Incorporation, Corning, New York). Ten µL of blanks (deionized water) and standards of Bovine Serum Albumin (Sigma, St. Louis, Missouri) ranging in concentrations of 0.125, 0.25, 0.5, 1.0 and 2.0 mg/mL were also added in triplicate.

Next, 20 µL of a mixture of 1 part copper sulphate (Sigma, St. Louis, Missouri) and 50 parts bicinchoninic acid (BCA, Sigma, St. Louis, Missouri) was added to each

well and mixed thoroughly. This mixture forms the principle of the test as proteins in the samples reduce the alkaline Cu^{2+} to Cu^{1+} in a concentration-dependent manner. The BCA is a highly specific chromogenic reagent for Cu^{1+} and the complex forms a purple colour with a maximum absorbance at 562 nm. The absorbance is directly proportional to the protein concentration in the samples. Therefore, after the $CuSO_4$ and BCA mixture was added to the wells and mixed thoroughly, the plate was incubated for 30 minutes at room temperature and the absorbance was read at 562 nm using the SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, California). The software on the plate reader plotted the standard curve and calculated the unknowns from the following equation for the line of the standard curve:

Urine Protein (mg/dL) =

$$\left[\frac{\text{Concentration of standard X A of unknown sample}}{\text{A of standard}}\right] \text{X dilution}$$

where, A = absorbance. Urine protein was then expressed per mg of urine creatinine.

3.4 Western Immunoblotting: Measurement of Protein Levels of Enzymes Involved in Eicosanoid Production and Protein Levels of PPARs in the Kidney

3.4.1 Lyophilization of Kidneys

Each half kidney was lyophilized in preparation for Western Immunoblotting of steady-state protein levels of COX-1, COX-2, $cPLA_2$, PPAR α, PPAR β and PPAR γ. Frozen kidneys were initially weighed, cut and placed in pre-weighed 15 mL test tubes with lids that had holes, immersed in liquid nitrogen and then placed on a freeze drying apparatus (Virtis, Model No 10-145MR-BA, Gardiner, New York). Tissue samples were dried until two consecutive equal weights were obtained. Dry kidneys were pulverized in

the test tube using a spatula, the lid was replaced with one without holes and the sample was stored at -80°C. All reagents used in the following procedures were purchased from Fisher Scientific, Mississauga, Ontario, unless otherwise stated.

3.4.2 Homogenization of Kidneys

Lyopholized kidney tissue was homogenized as per Cuozzo et al (2002). Thirty mg of frozen tissue from each lyopholized half kidney was weighed and placed in labeled tubes on ice and homogenized in 100 volumes of ice cold cytosolic homogenization buffer for a total of 60 seconds using a Polytron homogenizer (Brinkmann Instruments, Mississauga, Ontario). Cytosolic homogenization buffer contained 50 mM Tris (pH 10.89); 250 mM sucrose; 2 mM ethylene-diamine-tetraacetic acid (EDTA) (pH 7.6); 1 mM ethylene glycol-bis (β-aminoethyle ether) N,N,N',N'-tetracetic acid (EGTA) (pH 7.5); 50 μM NaF; 100 μM sodium orthovanadate; 25 μg/mL aprotinin; 25 μg/mL pepstatin; 25 μg/mL leupeptin; 1 μg/mL soybean trypsin inhibitor; 10 mM β-mercaptoethanol; and 144 μM 4-(2-aminoethyl) benzene sulfonyl fluoride (ABSF). Homogenates were transferred into 5 mL tubes and centrifuged at 100,000 g for 35 minutes at 4°C using the Beckman L5-50B ultracentrifuge (Mississauga, Ontario). The supernate fraction (cytosolic) was collected and stored immediately at -80°C. Using a glass rod and the Vortex-Genie 2 (VWR Scientific Products, West Chester, Pennsylvania), the remaining pellet was resuspended in 15 volumes of particulate homogenization buffer containing 1% Triton X-100, stored on ice for 10 minutes and centrifuged again at 100,000 g for 35 minutes at 4°C. The supernate fraction (particulate)

was collected and stored immediately at -80°C. The remaining pellet was resuspended in 0.5 mL particulate homogenization buffer and also stored at -80°C.

3.4.3 Microassay for Total Protein Determination

Total protein of experimental and control kidney homogenate cytosolic and particulate fractions was determined using the Bradford Method (Bradford, 1976). Using a Costar 96-well microplate (Conring Incorporation, Corning, New York), wells were labeled as blank, standard or sample. Standard concentrations of 0.05, 0.10, 0.20, 0.30 and 0.50 mg/mL were made using Bovine Serum Albumin (Sigma, St. Louis, Missouri). Ten µL of blank, standard and 20X diluted (with deionized water) cytosolic or particulate fraction were added to wells in triplicate. Two hundred µL of room temperature Bradford Reagent (Sigma, St. Louis, Missouri) was added to each well and mixed on an orbital shaker (Fisher Scientific, Fair Lawn, New Jersey, Model No. 361) for approximately 15 minutes until there was no precipitate. The plate was read at 595 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporation, Sunnyvale, California).). The software on the plate reader plotted the standard curve and calculated the unknowns from the following equation for the line of the standard curve:

Protein Concentration (µg/µL) =

$$\left[\frac{\text{Concentration of standard X A of unknown sample}}{\text{A of standard}} \right] \text{X dilution}$$

where, A = absorbance.

3.4.4 Western Immunoblotting

Steady-state protein levels of COX-1, COX-2, $cPLA_2$, PPAR α, PPAR β, and PPAR γ were measured using one-dimensional sodium dodecyl sulfate (SDS) gel electrophoresis. This allows for the separation of the complex mixture of proteins according to the protein,s migratory response to an electrical field through pores in a gel matrix. The combination of gel pore size and protein size determines the migration rate of each protein. For a complete summary of conditions specific for each protein of interest please see Table 5.

Cytosolic and particulate kidney fractions were first analyzed for the presence of the specific proteins of interest. Analysis of the pellet fraction was inhibited by the inability to dissolve particles in the supernatant fraction, which interfered with total protein measurements. $cPLA_2$ and PPAR γ were found in the cytosolic and particulate fractions. COX-1, COX-2, PPAR α and PPAR β were found in the particulate fraction only. Please refer to Figure 2 for Western Immunoblotting images of these proteins in their respective kidney homogenate fractions. Cytosolic and particulate fractions were treated with a 2X or 3X sample buffer, depending on the total protein concentration of the kidney homogenate fraction and the amount of total protein required to analyze the steady-state level of the specific protein of interest (the lower the protein concentration the more concentrated the sample buffer used). Dose response curves were generated to determine the level of protein required (Figures 3 to 10). To ensure accuracy, the amount of sample protein used was taken from the mid-range of the dose response curve. Please see Tables 6 to 8 for a list of kidney fraction protein concentrations and calculations used

Table 5 Western Immunoblotting: Summary of conditions for detection of proteins of interest.

Fraction and Primary Antibody	Protein to Load (μg)	Primary Antibody Concentration	First Washing	Secondary Antibody [c]	Secondary Antibody Concentration	Second Washing	Chemiglo™ Mixture	Develop (min)
Cytosol $cPLA_2$ [a]	16	1:250 2% SMP	3 X 5 min	Anti-rabbit	1:20,000	3 X 5 min	1A:1B:4H_2O	30
Particulate $cPLA_2$	16	1:250 2% SMP	3 X 5 min	Anti-rabbit	1:20,000	3 X 5 min	1A:1B:4H_2O	30
Particulate COX-1 [b]	16	1:250 2% SMP	3 X 10 min	Anti-mouse	1:20,000	3 X 10 min	1A:1B:4H_2O	30
Particulate COX-2 [b]	16	1:250 2% SMP	3 X 10 min	Anti-rabbit	1:20,000	3 X 10 min	1A:1B:4H_2O	30
Particulate PPAR α [b]	16	1:1000 5% SMP	3 X 10 min	Anti-rabbit	1:2000	5 X 10 min	1A:1B	5
Particulate PPAR β [a]	16	1:2500 2% SMP	4 X 10 min	Anti-goat	1:100,000	4 X 10 min	1A:1B	3
Cytosol PPAR γ [a]	16	1:200 5% SMP	3 X 10 min	Anti-rabbit	1:1000	5 X 10 min	1A:1B	2.5
Particulate PPAR γ	20	1:200 5% SMP	3 X 10 min	Anti-rabbit	1:1000	5 X 10 min	1A:1B	5

A = substrate A (Chemiglo™ West Luminol/Enhancer Solution, Fisher Scientific, Fair Lawn, New Jersey)
B = substrate B (Chemiglo™ West Stable Peroxide Solution, Fisher Scientific, Fair Lawn, New Jersey)
SMP = skim milk powder, supplied by Safeway, Winnipeg, Manitoba
[a] Supplied by Santa Cruz Biotechnology, Santa Cruz, California
[b] Supplied by Cayman Chemical, Ann Arbor, Michigan
[c] Supplied by Sigma, Saint Louis, Missouri

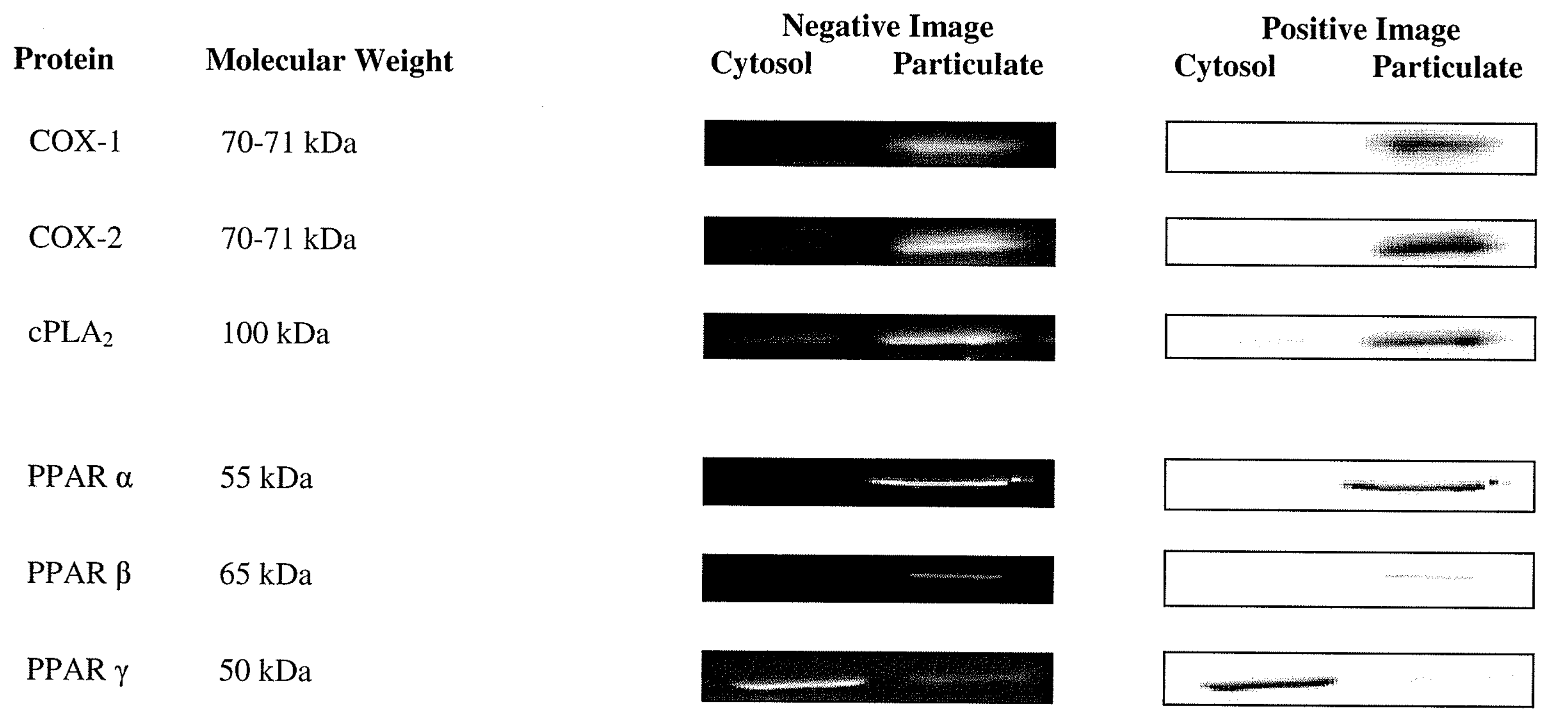

Figure 2 Western immunoblotting analysis for the presence of protein in kidney homogenate fractions. Samples are representative of lnCTRL. Negative and positive images are of the same sample.

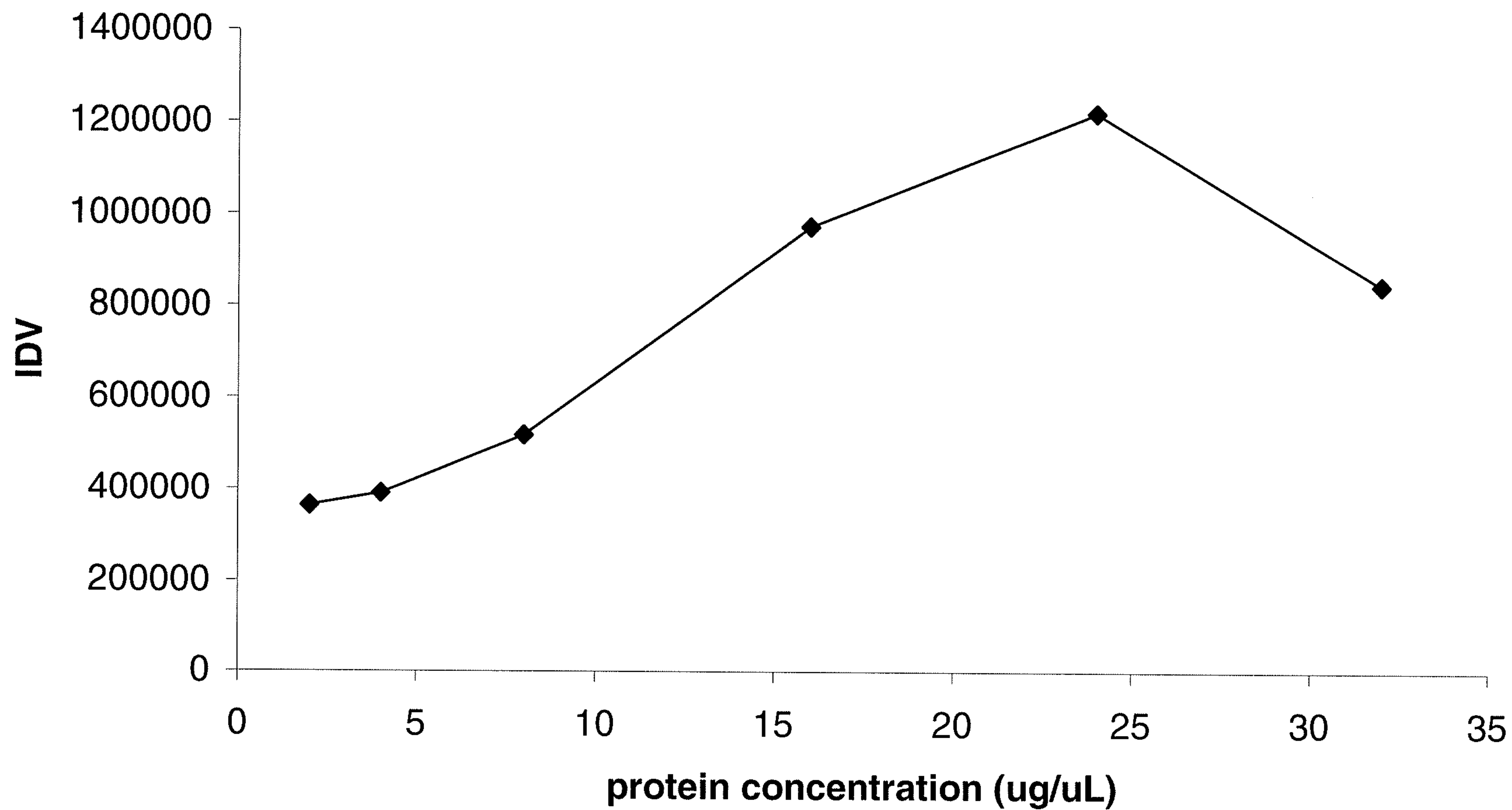

Figure 3 Cytosol $cPLA_2$ dose response curve

IDV = Integrated Density Value = Σ (each pixel value – background value)

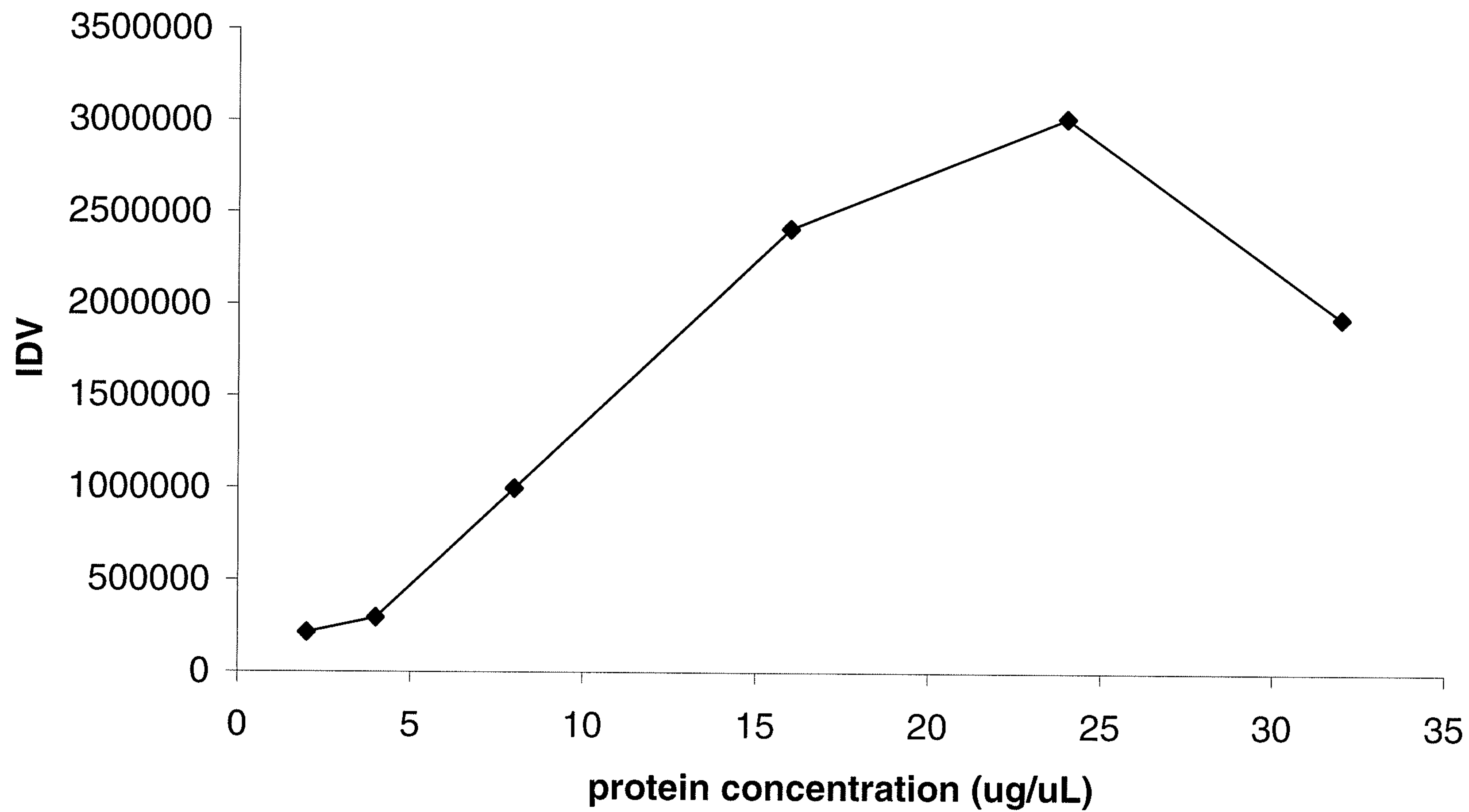

Figure 4 Particulate $cPLA_2$ dose response curve

IDV = Integrated Density Value = Σ (each pixel value – background value)

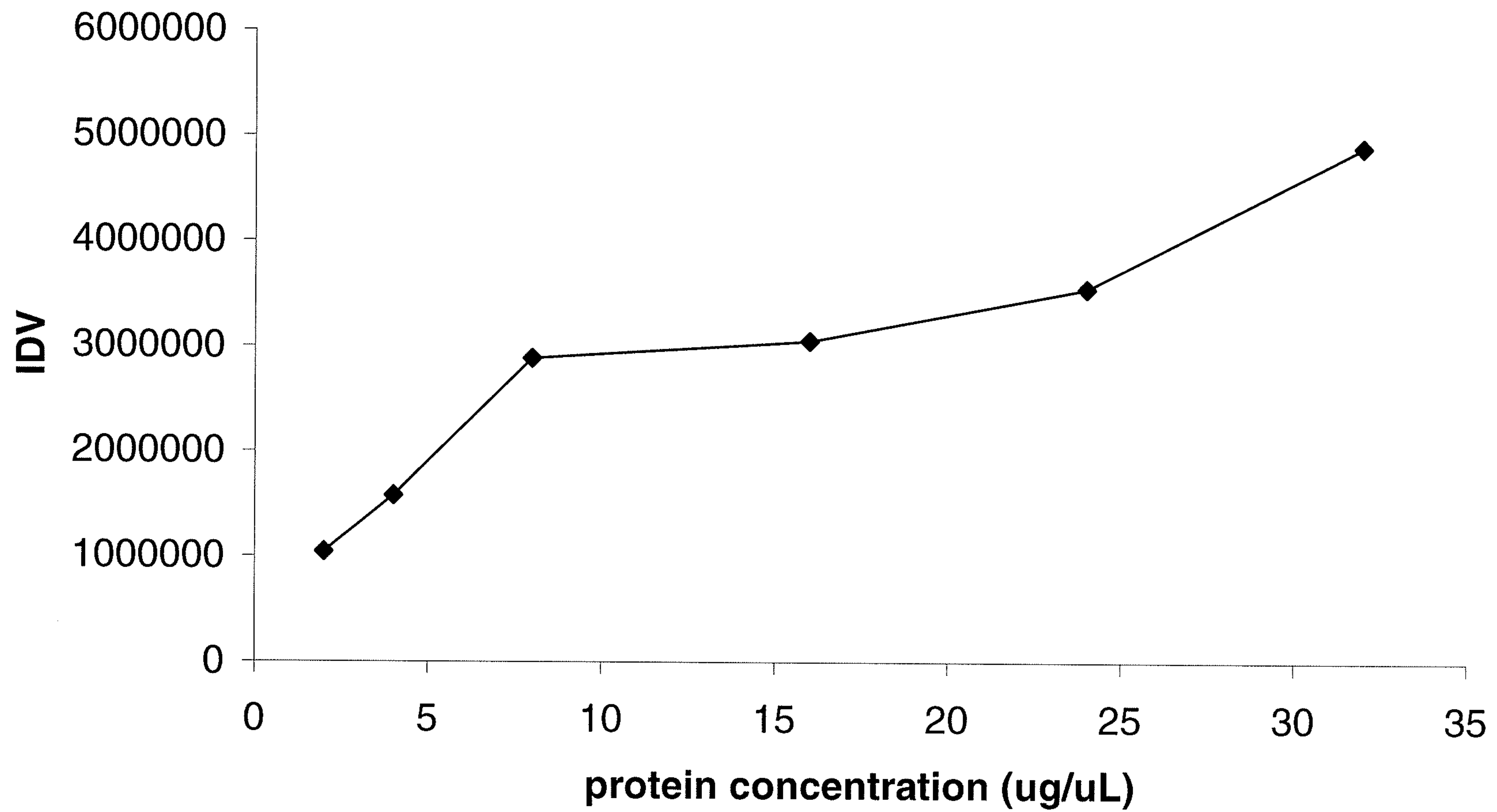

Figure 5 Particulate COX-1 dose response curve

IDV = Integrated Density Value = Σ (each pixel value – background value)

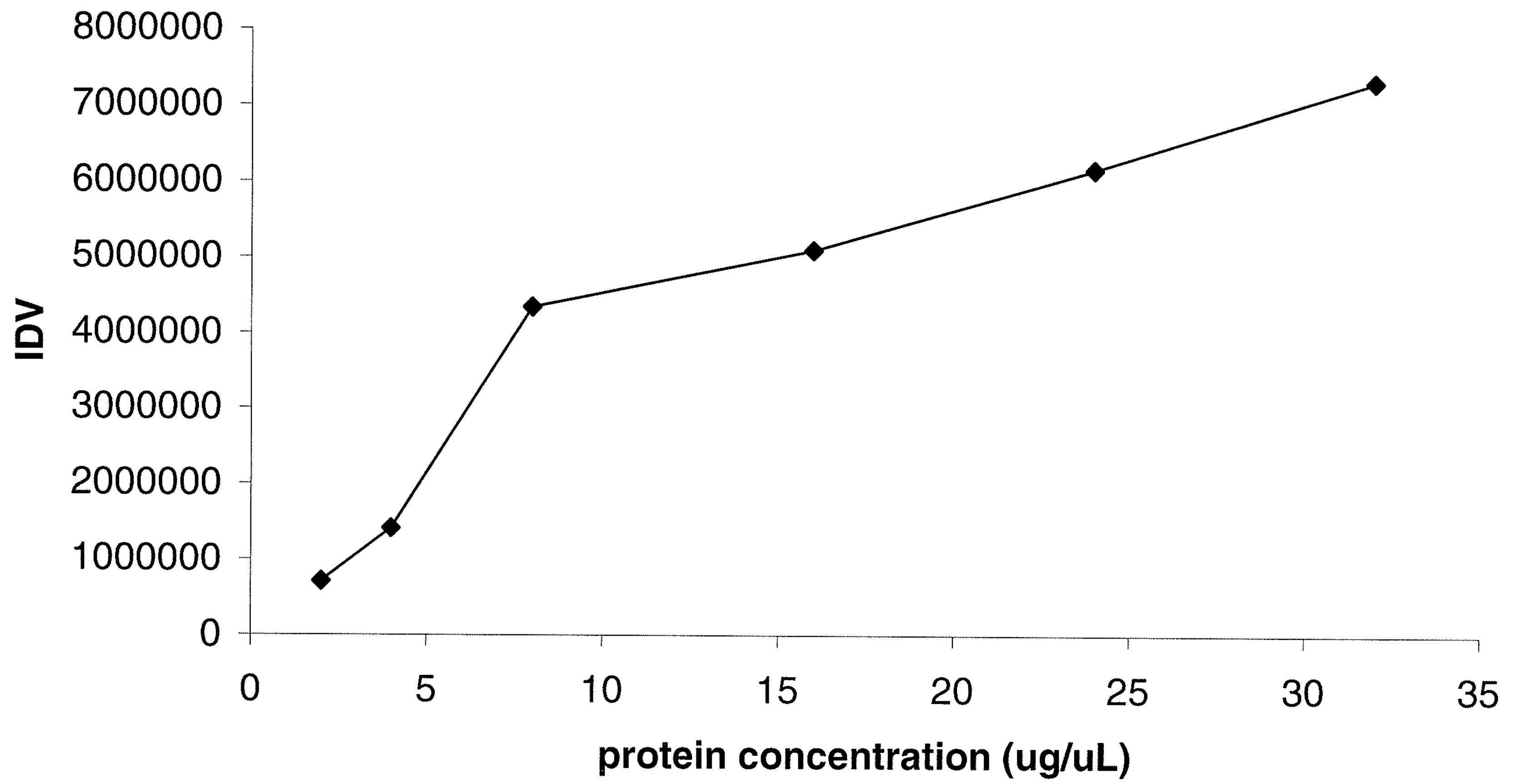

Figure 6 Particulate COX-2 dose response curve

IDV = Integrated Density Value = Σ (each pixel value – background value)

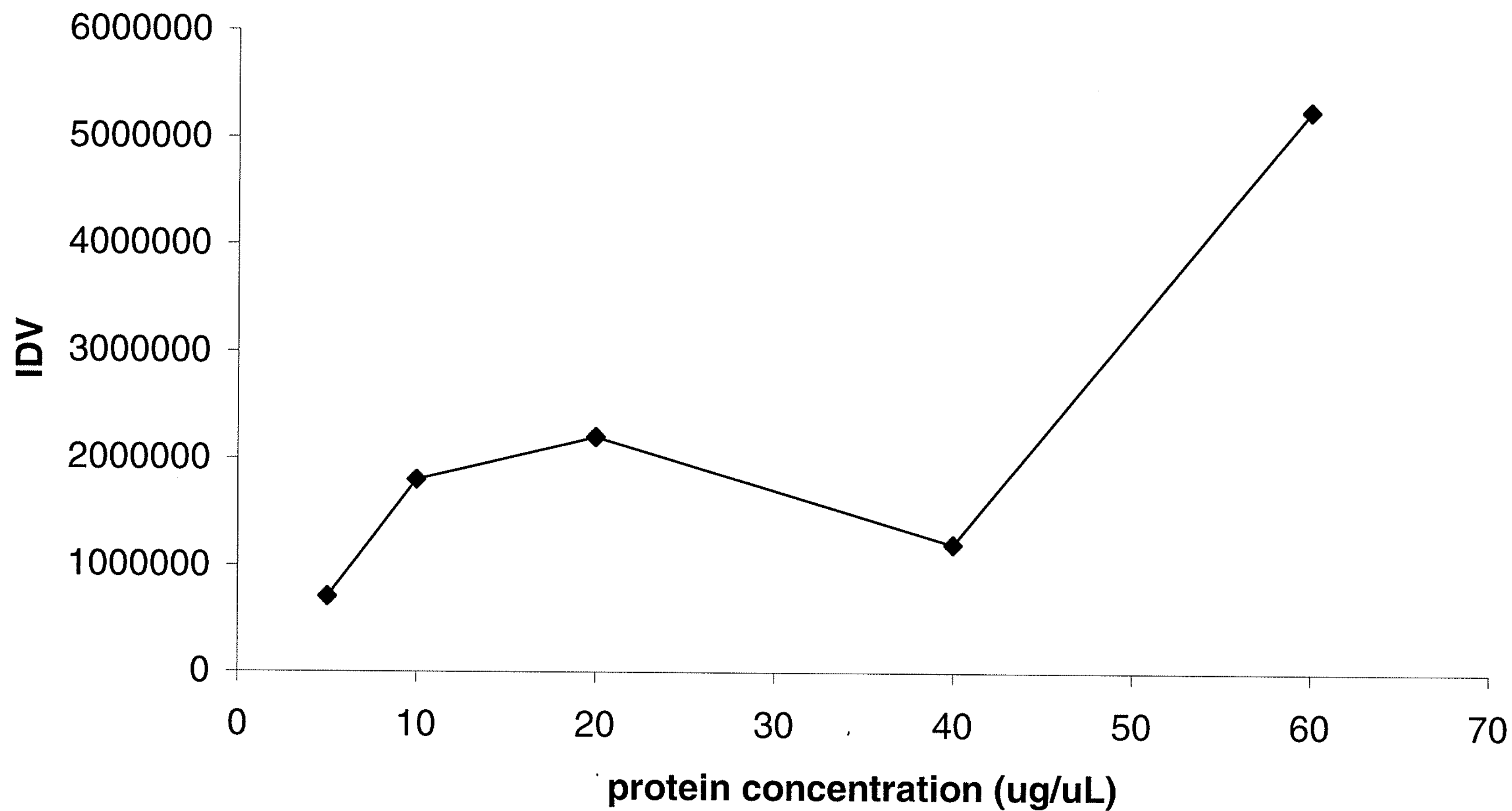

Figure 7 Particulate PPAR α dose response curve

IDV = Integrated Density Value = Σ (each pixel value – background value)

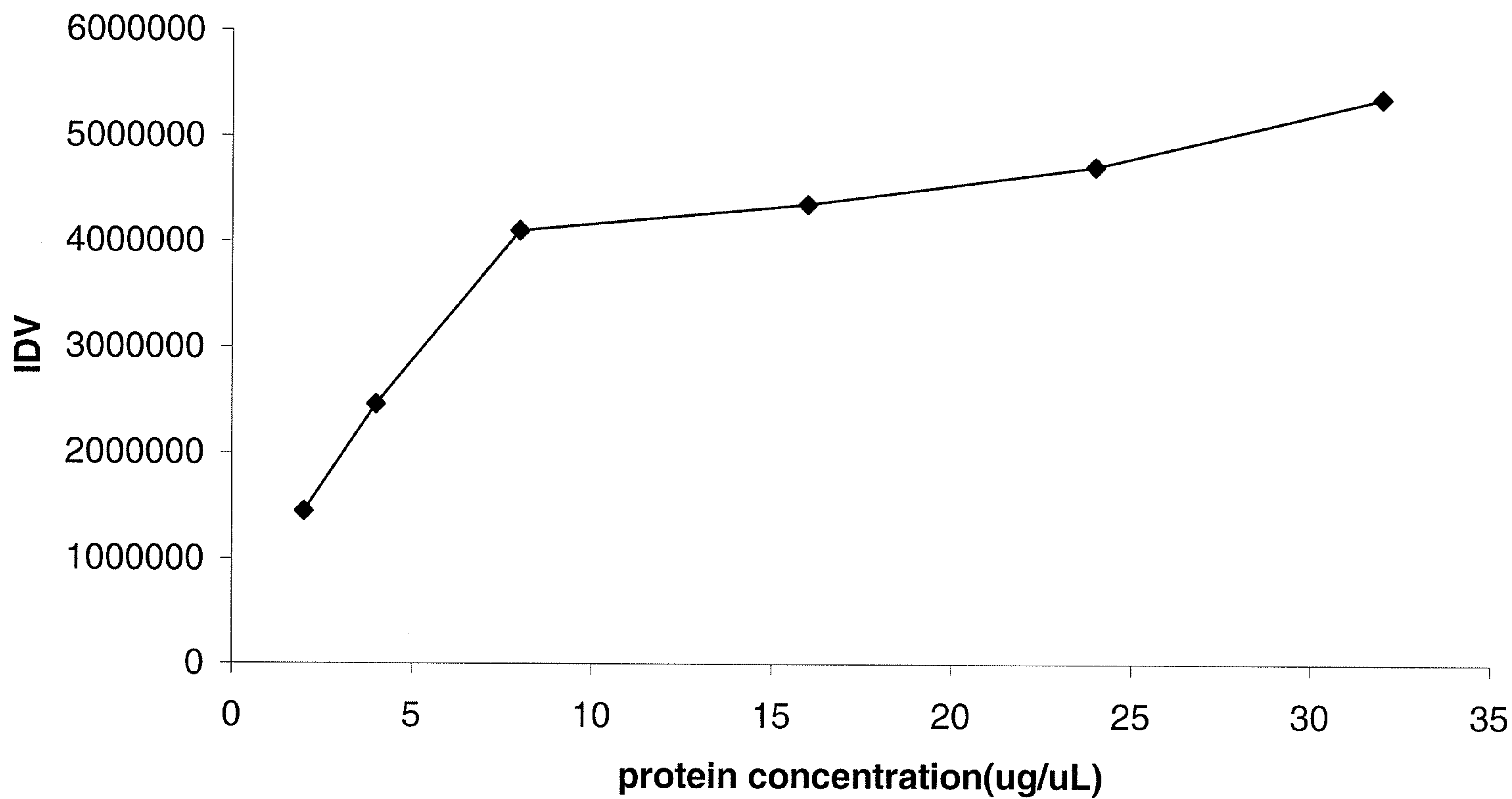

Figure 8 Particulate PPAR β dose response curve

IDV = Integrated Density Value = Σ (each pixel value – background value)

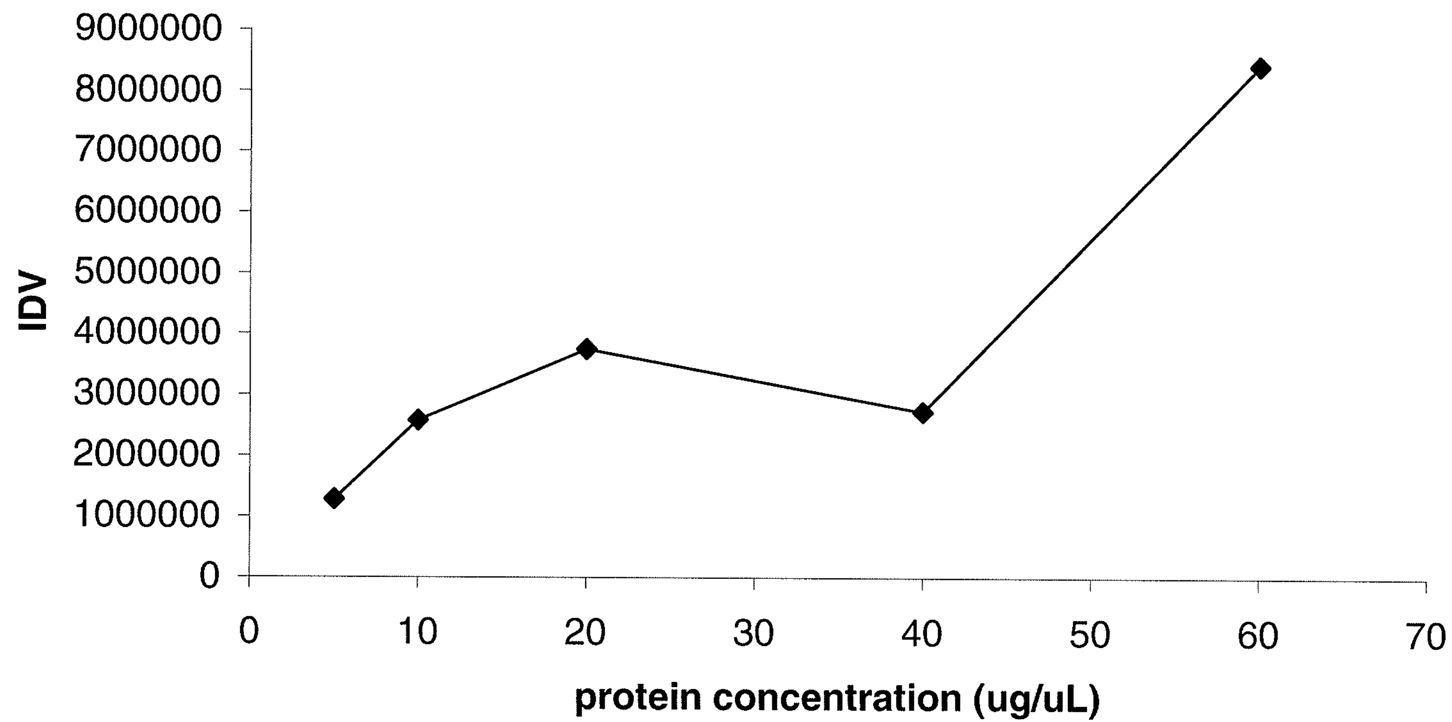

Figure 9 Cytosol PPAR γ dose response curve

IDV = Integrated Density Value = Σ (each pixel value – background value)

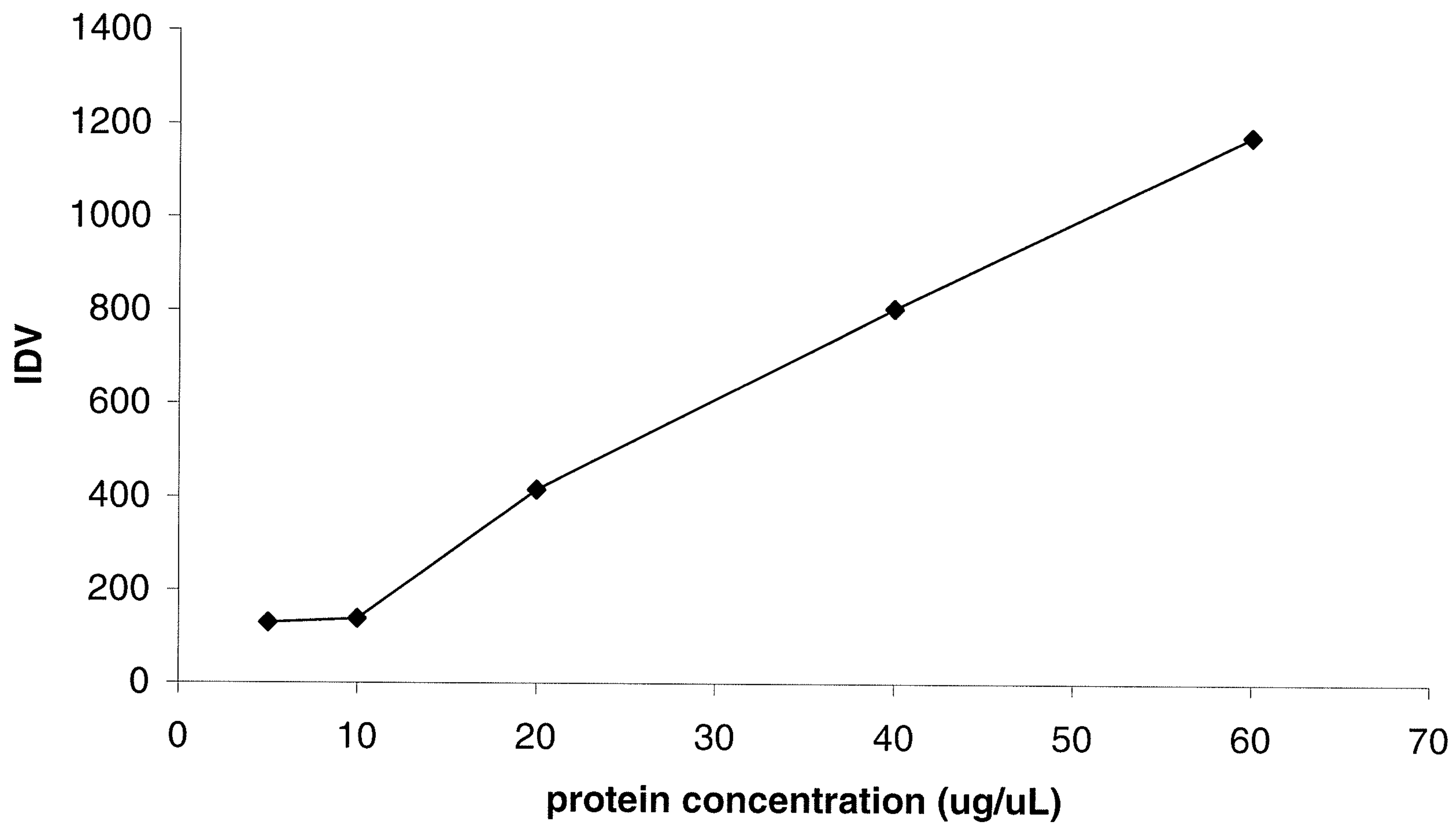

Figure 10 Particulate PPAR γ dose response curve

IDV = Integrated Density Value = Σ (each pixel value – background value)

Table 6 Cytosol protein concentrations and calculations for preparation of 16 μg sample.

Sample	Protein Concentration (μg/uL)	Volume sample to make 16ug (μL)	Volume Water (μL)	Volume 2X Buffer (μL)
faCLA				
1	4.48	3.57	3.43	7.00
7	4.44	3.60	3.40	7.00
13	3.66	4.37	2.63	7.00
19	3.16	5.06	1.94	7.00
25	3.90	4.10	2.90	7.00
31	5.12	3.13	3.88	7.00
37	4.34	3.69	3.31	7.00
43	3.42	4.68	2.32	7.00
49	4.18	3.83	3.17	7.00
faCTRL				
2	4.20	3.81	3.19	7.00
8	4.06	3.94	3.06	7.00
14	4.18	3.83	3.17	7.00
20	4.34	3.69	3.31	7.00
26	3.82	4.19	2.81	7.00
32	4.10	3.90	3.10	7.00
38	2.70	5.93	1.07	7.00
44	4.36	3.67	3.33	7.00
50	4.42	3.62	3.38	7.00
lnCLA				
4	3.82	4.19	2.81	7.00
10	4.30	3.72	3.28	7.00
16	2.68	5.97	1.03	7.00
22	2.68	5.97	1.03	7.00
28	4.48	3.57	3.43	7.00
34	4.40	3.64	3.36	7.00
40	2.50	6.40	0.60	7.00
46	3.80	4.21	2.79	7.00
52	3.94	4.06	2.94	7.00
lnCTRL				
5	4.28	3.74	3.26	7.00
11	4.26	3.76	3.24	7.00
17	3.84	4.17	2.83	7.00
23	4.54	3.52	3.48	7.00
29	2.80	5.71	1.29	7.00
35	3.50	4.57	2.43	7.00
41	3.92	4.08	2.92	7.00
47	3.20	5.00	2.00	7.00
53	4.56	3.51	3.49	7.00

Table 7 Particulate protein concentrations and calculations for preparation of 16 μg sample.

Sample	Protein Concentration (μg/uL)	Volume sample to make 16ug (μL)	Volume Water (μL)	Volume 3X Buffer (μL)
faCLA				
1	5.62	2.85	4.15	7.00 (2X)
7	4.12	3.88	6.12	5.00
13	5.36	2.99	4.01	7.00 (2X)
19	5.26	3.04	3.96	7.00 (2X)
25	3.76	4.26	5.74	5.00
31	5.06	3.16	6.84	5.00
37	3.88	4.12	5.88	5.00
43	2.96	5.41	4.59	5.00
49	4.62	3.46	6.54	5.00
faCTRL				
2	4.38	3.65	6.35	5.00
8	5.08	3.15	6.85	5.00
14	3.92	4.08	5.92	5.00
20	4.12	3.88	6.12	5.00
26	3.48	4.60	5.40	5.00
32	4.12	3.88	6.12	5.00
38	4.12	3.88	6.12	5.00
44	5.26	3.04	3.96	7.00 (2X)
50	4.44	3.60	6.40	5.00
lnCLA				
4	4.92	3.25	6.75	5.00
10	4.40	3.64	6.36	5.00
16	4.88	3.28	6.72	5.00
22	4.02	3.98	6.02	5.00
28	3.80	4.21	5.79	5.00
34	5.60	2.86	4.14	7.00 (2X)
40	3.88	4.12	5.88	5.00
46	6.00	2.67	4.33	7.00 (2X)
52	5.26	3.04	6.96	5.00
lnCTRL				
5	4.02	3.98	6.02	5.00
11	4.04	3.96	6.04	5.00
23	4.82	3.32	6.68	5.00
29	4.56	3.51	6.49	5.00
35	5.64	2.84	4.16	7.00 (2X)
41	4.48	3.57	6.43	5.00
47	5.78	2.77	4.23	7.00 (2X)
53	4.48	3.57	6.43	5.00
59	5.14	3.11	3.89	7.00 (2X)

Table 8 Particulate protein concentrations and calculations for preparation of 20 μg sample.

Sample	Protein Concentration (μg/uL)	Volume sample to make 20ug (μL)	Volume Water (μL)	Volume 3X Buffer (μL)
faCLA				
1	5.62	3.56	6.44	5.00
7	4.12	4.85	5.15	5.00
13	5.36	3.73	6.27	5.00
19	5.26	3.80	6.20	5.00
25	3.76	5.32	4.68	5.00
31	5.06	3.95	6.05	5.00
37	3.88	5.15	4.85	5.00
43	2.96	6.76	3.24	5.00
49	4.62	4.33	5.67	5.00
faCTRL				
2	4.38	4.57	5.43	5.00
8	5.08	3.94	6.06	5.00
14	3.92	5.10	4.90	5.00
20	4.12	4.85	5.15	5.00
26	3.48	5.75	4.25	5.00
32	4.12	4.85	5.15	5.00
38	4.12	4.85	5.15	5.00
44	5.26	3.80	6.20	5.00
50	4.44	4.50	5.50	5.00
lnCLA				
4	4.92	4.07	5.93	5.00
10	4.40	4.55	5.45	5.00
16	4.88	4.10	5.90	5.00
22	4.02	4.98	5.02	5.00
28	3.80	5.26	4.74	5.00
34	5.6	3.57	6.43	5.00
40	3.88	5.15	4.85	5.00
46	6	3.33	6.67	5.00
52	5.26	3.80	6.20	5.00
lnCTRL				
5	4.02	4.98	5.02	5.00
11	4.04	4.95	5.05	5.00
23	4.82	4.15	5.85	5.00
29	4.56	4.39	5.61	5.00
35	5.64	3.55	6.45	5.00
41	4.48	4.46	5.54	5.00
47	5.78	3.46	6.54	5.00
53	4.48	4.46	5.54	5.00
59	5.14	3.89	6.11	5.00

in sample preparation. The 2X sample buffer contained 62.5 mM Tris-HCl (pH 6.8); 10% (w/v) glycerol; 2% (w/v) SDS; 7.1 M β-mercaptoethanol; and 0.05% (w/v) bromophenol blue. The 3X sample buffer contained 150 mM Tris-HCl (pH 6.8); 1.5% (w/v) glycerol; 3% (w/v) SDS; 10.7 M β-mercaptoethanol; and 0.08% (w/v) bromophenol blue. After samples were prepared, they were heated at 100°C for 5 minutes to denature proteins, placed in an Eppendorf 5417C centrifuge at 7000 rpm for 1 minute (Brinkmann Instruments, Mississauga, Ontario), and placed on ice. Using the Hoefer miniVE verticle electrophoresis system (Amersham Biosciences, Baie d'Urfé, Québec) a 7.5% separating gel was made and poured into the casting gel apparatus to ¾ full and topped with deionized water. Five mL of separating gel contained 2.67 mL deionized water, 1.25 mL 1.5M Tris-HCl (pH 8.8), 50 µL 10% (w/v) SDS, 1.0 mL acrylamide : bis-acrylamide 29:1 40% stock solution, 10% (w/v) ammonium persulfate (APS), 5 µL N,N,N',N'-tetramethylethylenediame (TEMED). Once solidified, the deionized water was poured off and the separating gel was topped with a stacking gel to which a 15 well comb was placed. Five mL of stacking gel contained 3.25 ml deionized water, 1.25 mL 0.5M Tris-HCl (pH 6.8), 50 µL 10% SDS, 0.5 mL acrylamide : bis-acrylamide 29:1 40% stock solution, 25 µL 10% APS, 5 µL TEMED.

Once the stacking gel had solidified, the casting gel apparatus was placed in an electrophoretic chamber. The upper compartment of the casting gel apparatus and the chamber were filled with 1X running buffer and the comb was removed from the stacking gel. To make the 1X running buffer a 10X, running buffer was first made by mixing 29.0 g Tris base with 144.0 g glycine and 10.0 g SDS, and filling to 1L with deionized water. This stock solution was stored at room temperature. One hundred mL of this stock

solution was then diluted with 900 mL of deionized water to end up with a 1X running buffer. The appropriate amount of protein was then loaded into each well after each well had been washed with running buffer. Two wells were used to load a standardized amount of protein (same sample loaded to each gel) so that the average density of each band produced could be used to calculate unknown band densities as a percentage of the standard. A molecular weight marker (BenchMark^{TM}Prestained Protein Ladder, Cat # 10748-010, Invitrogen Life Technologies, Carlsbad, California) was also loaded into one well on each gel to ensure that the protein band of interest was at the specific molecular weight after immunoblotting. The apparatus was then attached to a power source (Amersham Pharmacia Biotech EPS 301, Little Chalfont, Buckinghamshire, England) and ran at 200V at room temperature until just before the bands come off the gel (approximately 1.5 hours).

After electrophoresis, proteins were transferred from the gel to polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech Hybond-P, cat # RPN303F, Little Chalfont, Buckinghamshire, England). Membranes were first cut to the appropriate size and labeled with date and gel number. The lower left corner was cut in order to indicate the first lane. The membrane was soaked in high performance liquid chromatography (HPLC) grade methanol for 10 seconds, then in water for 7 minutes and finally in 1X transfer buffer for equilibration until the gel was ready for protein transfer. Before protein transfer, the gel was also equilibrated for 10 minutes in 1X transfer buffer. To make the 1X transfer buffer, a 10X transfer buffer was first made by mixing 29.0 g Tris base with 144.0 g of glycine and filling to 1L with deionized water. This stock solution was stored at room temperature. One hundred mL of this 10X transfer buffer

stock was mixed with 200 mL of HPLC grade methanol and 700 mL of deionized water to end up with a 1X transfer buffer. This solution was stored at 4°C.

The transfer apparatus was set up with the gels and the membranes placed in such a way as to form a "sandwich" that contained a sponge then a piece of filter paper, topped with the gel, which is topped with the membrane, which is topped with a piece of filter paper. Placed on the top of all this were 6 more sponges, all of which were soaked in 1X transfer buffer. The side of the gel touching the paper subjectively becomes the cathode side of the gel. Proteins are usually negatively charged in transfer buffer and therefore, will move towards the positively charged anode. The transfer apparatus was then placed in a transfer chamber that was filled with cold, distilled water such that the membrane to which proteins were to be transferred faced the anode side of the chamber. The transfer apparatus was filled with transfer buffer. The transfer occurred over 2 hours at 375mA at 4°C.

Once the transfer was complete, the membrane was removed from the transfer apparatus and placed in 5% skim milk solution, prepared in a Tris base solution (TBS) with 0.1% enzyme grade Tween, (TBS/Tween), for one hour on a rocker (Boekel Scientific Rocker II, model 260350, Feastville, Pennsylvania) at room temperature. The TBS/Tween solution was prepared by mixing 24.2 g of tris base with 80 g of NaCl and filling to 800 mL. The pH was adjusted to 7.6 using a 1:1 dilution of HCl. Ten mL of enzyme grade Tween was then added followed by enough deionized water to make 1000 mL of solution. Finally, 100 mL of this solution was diluted with 900 mL deionized water. The blocking process blocks all the remaining protein sites. Primary antibody solution consisting of either a 2% or 5% skim milk solution was prepared in TBS/Tween

and poured over the membrane and placed on a shaker in a 4°C refrigerator overnight. The concentration of the primary antibody and skim milk powder used was specific for the type of protein to be detected. Following the treatment with the primary antibody, the membrane was washed with TBS/Tween for a length of time specified by the type of protein to be detected. Next, secondary antibody that was conjugated to horseradish peroxidase was made using TBS/TWEEN. The type of secondary antibody and the concentration of skim milk powder used was specific for the type of protein to be detected. This secondary antibody solution was then added to the membrane and placed on a rocker for one hour at room temperature. After one hour, the membrane was washed again with TBS/Tween for a length of time specified by the type of protein to be detected. The antigen/primary antibody/secondary antibody/enzyme complex bound to the membrane was then detected by chemiluminescent imaging.

Detection solutions using ChemiGlow™, a chemiluminescent substrate for horseradish peroxidase, was mixed approximately 15 minutes before the membranes were ready to be developed and placed in a dark place until ready for use. After the last wash, TBS/Tween was poured off the membrane and the membrane was placed on a piece of saran wrap. Using a Pasteur pipette, the ChemiGlow™ chemiluminescent substrate solution was evenly distributed over the surface of the membrane, which was left to sit for 5 minutes. The ChemiGlow™ chemiluminescent substrate solution was drained off and the membrane was placed on the top half of a new piece of saran wrap. The bottom half of the saran wrap was folded over the membrane, ensuring there were no folds or air bubbles in the saran covering. The membrane was then developed using Fluorchem™ FC digital imaging system (Alpha Innotech Corporation, San Leandro,

California) using the length of time specific for the type of protein to be detected. Once the bands were imaged, the optical densities (OD) of the bands was measured using the Fluorchem^{TM} FC software and the final value in OD/μg of protein was used to calculate the OD/ug of protein of the unknowns as a percentage of the OD/ug of protein of the standard. This percentage of standard was used for statistical analysis. OD/ug of protein was determined by first calculating the integrated density value (IDV). The IDV is equal to the sum of each pixel value minus the background value. The average value was then calculated by dividing the IDV by the size (in pixels) of the region in which the band is enclosed.

3.5 Eicosanoid Analysis

An attempt was made to measure *ex vivo* COX-1 and COX-2 enzyme activity and the levels of PGE_2, TXB_2, (a stable metabolite of TXA_2) and 6-keto $PGF_{1\alpha}$, (a stable metabolite of PGI_2) in lyopholized kidney tissue using competitive protein binding kits purchased from Cayman Chemical (Ann Arbor, Michigan). The assay is based on the competition between the eicosanoid of interest and an eicosanoid tracer (the eicosanoid of interest bound to an acetylcholinesterase conjugate) for a limited number of eicosanoid specific antibody binding sites. Because the concentration of the tracer remains constant while the concentration of eicosanoid in kidney tissue samples vary, the amount of tracer that is able to bind to the antibody is inversely proportional to the concentration of the eicoasanoid in the sample. The antibody-eicosanoid complex then binds to a secondary antibody that is attached to the wall of wells in a 96 well plate. The wells are washed to remove any unbound reagents and then a reagent, which contains the substrate to the

acetylcholinesterase conjugate, is added to the wells. The product of the enzymatic reaction has a distinct colour which absorbs strongly at 412 nm. The intensity of the colour is determined spectrophotometrically, using a microplat reader, and is proportional to the amount of eicosanoid tracer and therefore, is inversely proportional to the amount of eicosanoid in the sample.

Pre-testing was conducted using practice kidney tissue samples. Seventy mg of sample was homogenized, on ice, in 2 mL of cold Tyrodes's buffer (Sigma, St. Louis, Missouri) using a Polytron homogenizer (Brinkman Instruments, Mississauga, Ontario) and aliquoted into chilled 2 mL eppendorf centrifuge tubes. Two hundred μL of homogenate was aliquoted into 6 chilled 2 mL eppendorf centrifuge tubes and either no inhibitor or a chilled COX-1 inhibitor (SC-560, Cayman, Ann Arbor, Michigan) or a chilled COX-2 inhibitor (NS-398, Cayman, Ann Arbor, Michigan) was added to each set of six tubes. The final concentration of COX-1 inhibitor added was 0.3 μM while the final concentration of COX-2 inhibitor added was 50 μM. Tubes were placed in a 37 °C water bath for 2.5, 5, 10, 20, 40 and 80 minutes after which time they were placed on ice and 800 μL of a 17.5 mM solution of chilled aspirin (Sigma, St. Louis, Missouri), dissolved in Tyrodes' buffer, was added to give a final aspirin concentration of 14 mM to stop the reaction. Samples were centrifuged (IEC Micromax, International Equipment Company, Needham Heights, Massachusetts) for 5 min at 10,000 g in a 4 °C walk-in refrigerator. The supernate fraction was drawn off and placed into clean, chilled 2 mL eppendorf centrifuge tubes. Samples were diluted using Tyrode's buffer at 3 different dilutions (400X, 1200X and 2400X) and frozen at -80 °C. Diluted samples were thawed on ice in preparation for the assay.

Due to time constraints and problems with the inability to reproduce results using enzyme inhibitors in the pre-testing stage, further attempts to obtain the eicosanoid data was abandoned by the investigator. To date, the details surrounding the inability to reproduce results are still being worked out by Dr. Aukema's lab and the investigator of this thesis continues to play a small part in working out these details.

3.6 Statistical Analysis

Differences between dietary treatment groups were analyzed using the SAS System for Windows Version 8 (SAS Institute, Cary, North Carolina). A two-way ANOVA was used to analyze all data, except Western Immunoblotting data, where a mixed model of analysis was performed. The main effects were diet, genotype and the interaction of diet and genotype for the two-way ANOVA. The fixed effects were diet, genotype and the interaction of diet and genotype and the random effect was homogenization day for the mixed model of analysis. A mixed model of analysis was performed for the Western Immunoblotting data because all kidney tissue samples were not homogenized on the same day and it was later found that on one particular day a problem had occurred with the homogenization process, likely due to altered pH of the particulate buffer, therefore, creating a day effect. These samples were re-homogenized and the particulate proteins analyzed again. Once samples from the bad day were removed, there was no significant day effect observed.

Raw data that was not normally distributed was transformed by taking the logarithm of the raw data. In cases where the logarithm data was not normally distributed, the arctangent of the raw data was also computed and all raw data and

transformed data where analyzed separately (i.e. raw, logarithm and arctangent data). In all cases where this occurred, the analysis results for the raw and transformed data yielded the same results. These results where then again confirmed using a non-parametric one-way ANOVA. Normality was judged according to the Kolmogorov-Smirnov Goodness of Fit test ($D > 0.05$ considered normally distributed) and the following distribution plots: 1) Box-&-Whisker, 2) Histogram and 3) Probability Plot. Tukey's post hoc test was used to determine differences between groups if a significant interaction was detected. Differences were considered significant at a $P < 0.05$. All data are reported as mean ± the standard error of the mean (SEM) for the raw data.

4 RESULTS

4.1 Assessment of Growth

4.1.1 Feed Intake

As can be seen in Figure 11, total feed intake for the 8-week study period was 43% greater for the obese *fa/fa* Zucker rats compared to lean Zucker rats (1538.5 ± 27.4 g versus 1077.7 ± 22.1 g, respectively, $P < 0.0001$).

4.1.2 Body Weight

As seen in Figure 12, final body weight was 58% greater for the obese *fa/fa* Zucker rats compared to lean Zucker rats (566.9 ± 9.6 g versus 357.7 ± 6.0 g, respectively, $P < 0.0001$). Obese *fa/fa* Zucker rats had a total weight gain of 382.4 ± 10.9 g while lean Zucker rats had a total weight gain of 216.8 ± 5.7 g.

4.2 Disease Progression

4.2.1 Kidney Weight

Figure 13 provides the final kidney weight for each experimental group. There was a diet by genotype interaction ($P = 0.0225$). Tukey's post hoc analysis revealed that obese *fa/fa* Zucker rats fed CLA had an 11% smaller kidney weight compared to obese *fa/fa* Zucker rats fed CTRL (3.3 ± 0.1 g versus 3.7 ± 0.1 g, respectively). Obese *fa/fa* Zucker rats fed CLA had a 14% larger kidney weight than lean Zucker rats fed CLA (3.3 ± 0.1 g versus 2.9 ± 0.1 g, respectively), while obese *fa/fa* Zucker rats fed CTRL had a 28% larger kidney weight compared to lean Zucker rats fed CTRL (3.7 ± 0.1 g versus 2.9 ± 0.1 g, respectively).

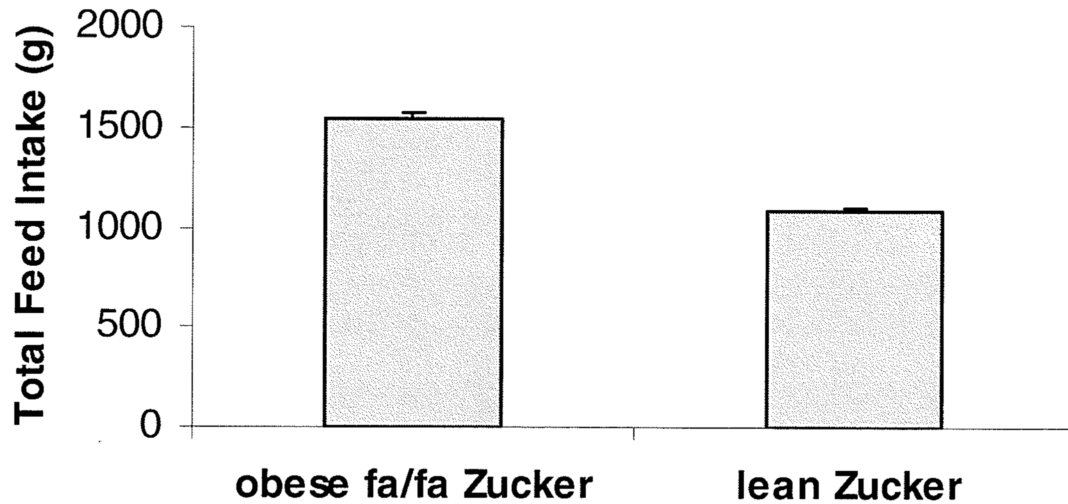

Figure 11 Total feed intake for the 8-week study period. Data is presented as mean ± SEM (n = 10). 2X2 ANOVA with $P < 0.05$ is considered significantly different. Genotype effect, $P < 0.0001$.

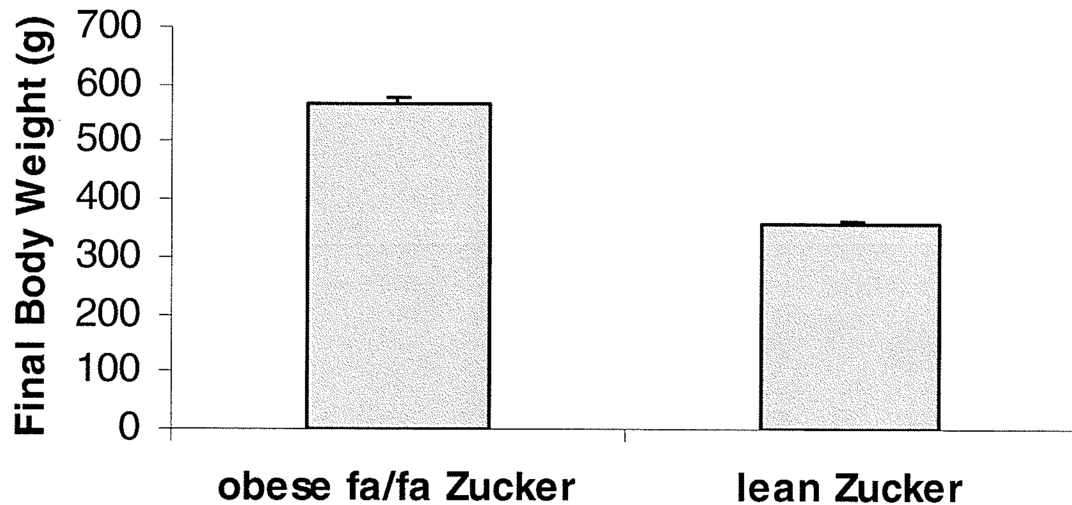

Figure 12 Final body weight. Data is presented as mean ± SEM (n = 10). 2X2 ANOVA with $P < 0.05$ is considered significantly different. Genotype effect, $P < 0.0001$.

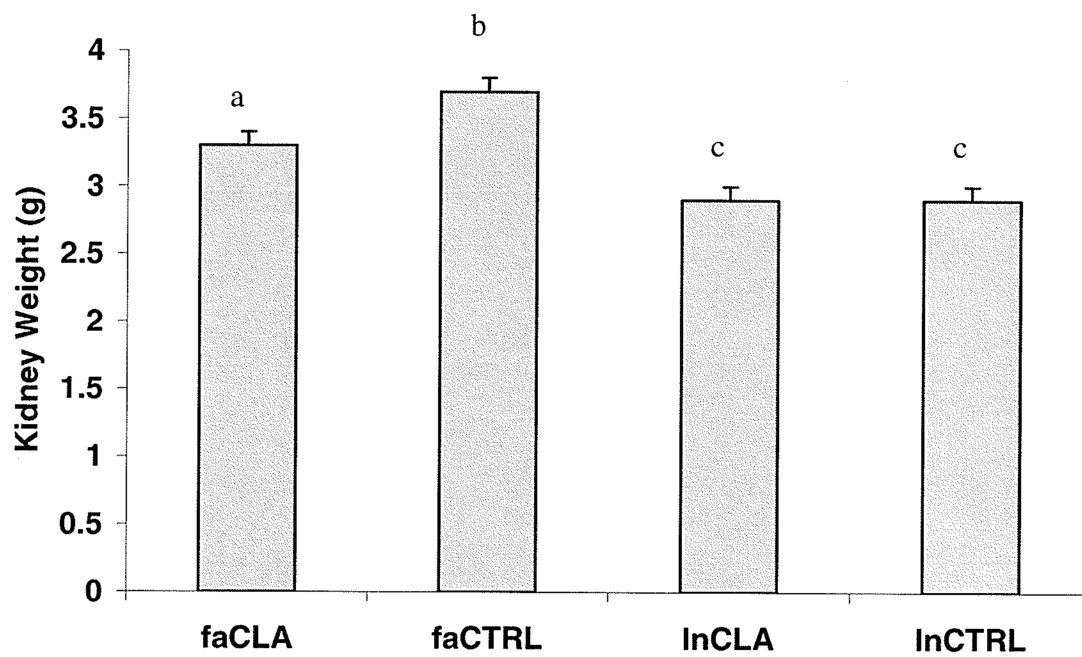

Figure 13 Kidney weight at 8 weeks. Data is expressed as mean ± SEM (n = 10). Columns with different letters are significantly different at $P < 0.05$ using Tukey's post hoc test. Diet by genotype interaction, $P = 0.0225$.

Figure 14 demonstrates the final kidney weight per 100 g of final body weight for each experimental group. Obese *fa/fa* Zucker rats had a 23% lower kidney to body weight ratio compared to lean Zucker rats (0.62 ± 0.01 g kidney weight/100 g final body versus 0.80 ± 0.01 g kidney weight/100 g final body weight, respectively, $P < 0.0001$).

4.2.2 Mean Glomerular Volume

Figure 15 demonstrates the MGV for each experimental group. There was a diet by genotype interaction ($P = 0.0024$). Tukey's post hoc analysis revealed that obese *fa/fa* Zucker rats fed CLA had a 28% smaller MGV compared to obese *fa/fa* Zucker rats fed CTRL (1.593 ± 0.103 $\mu m^3 \times 10^6$ versus 2.201 ± 0.112 $\mu m^3 \times 10^6$, respectively). Obese *fa/fa* Zucker rats fed CLA had a 30% larger MGV than lean Zucker rats fed CLA (1.593 ± 0.103 $\mu m^3 \times 10^6$ versus 1.230 ± 0.066 $\mu m^3 \times 10^6$, respectively), while obese *fa/fa* Zucker rats fed CTRL had an 84% larger MGV compared to lean Zucker rats fed CTRL (2.201 ± 0.112 $\mu m^3 \times 10^6$ versus 1.193 ± 0.056 $\mu m^3 \times 10^6$, respectively). Figures 16 to 19 are representative sections of renal tissue from CLA and CTRL fed obese *fa/fa* Zucker rats and lean Zucker rats after 8 weeks of dietary intervention.

4.3 Measurements of Kidney Function

4.3.1 Urine Volume

Table 9 summarizes measurements of kidney function assessed. Obese *fa/fa* Zucker rats had a 32% higher 12 hr urine volume than lean Zucker rats (7.22 ± 0.44 mL/12 hr versus 5.46 ± 0.47 mL/12 hr, respectively, $P < 0.0088$).

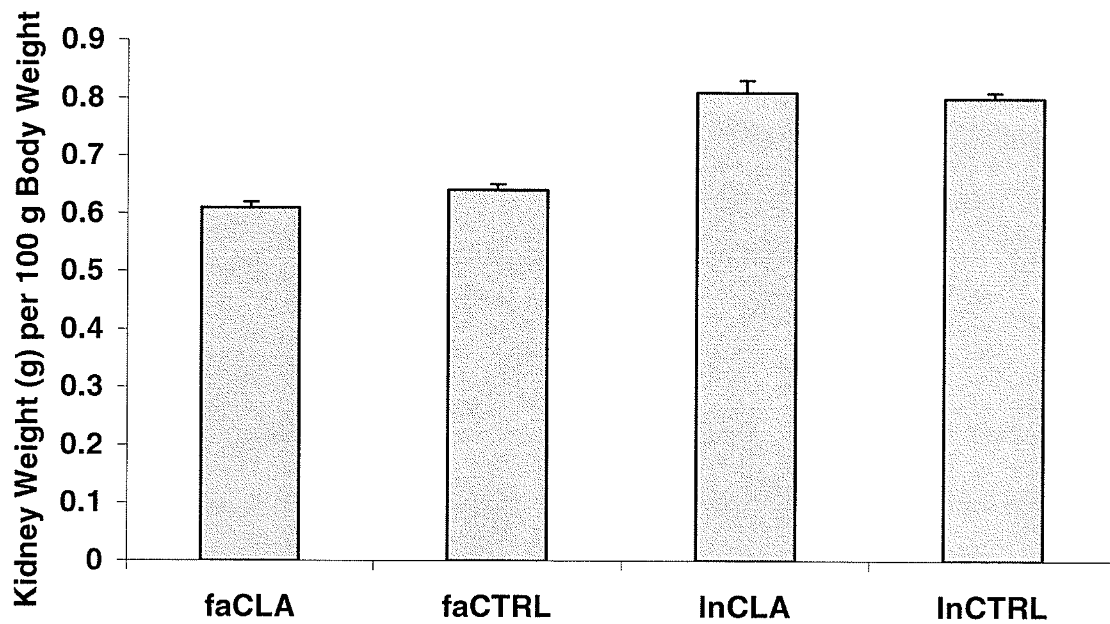

Figure 14 Kidney weight at 8 weeks expressed as 100 g of final body weight. Data is expressed as mean ± SEM (n = 10). 2X2 ANOVA with $P < 0.05$ is considered significantly different. Genotype, $P < 0.0001$.

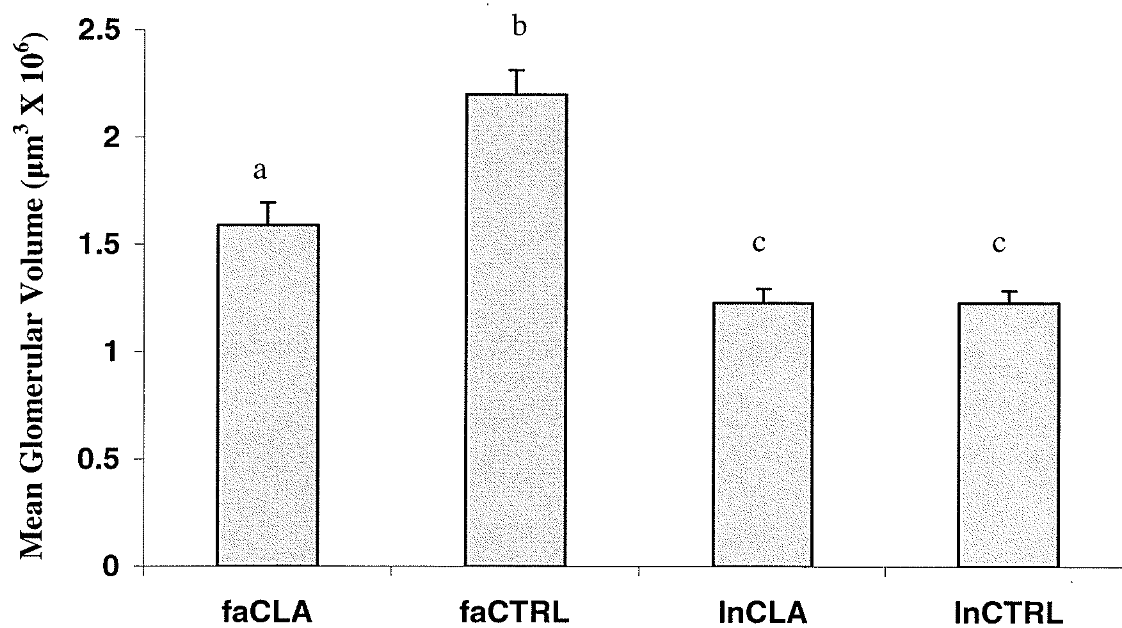

Figure 15 Mean glomerular volume at 8 weeks. Data is expressed as mean ± SEM (n = 10, except n = 9 for lnCTRL). Columns with different letters are significantly different at $P < 0.05$ using Tukey's post hoc test. Diet by genotype interaction, $P = 0.0024$.

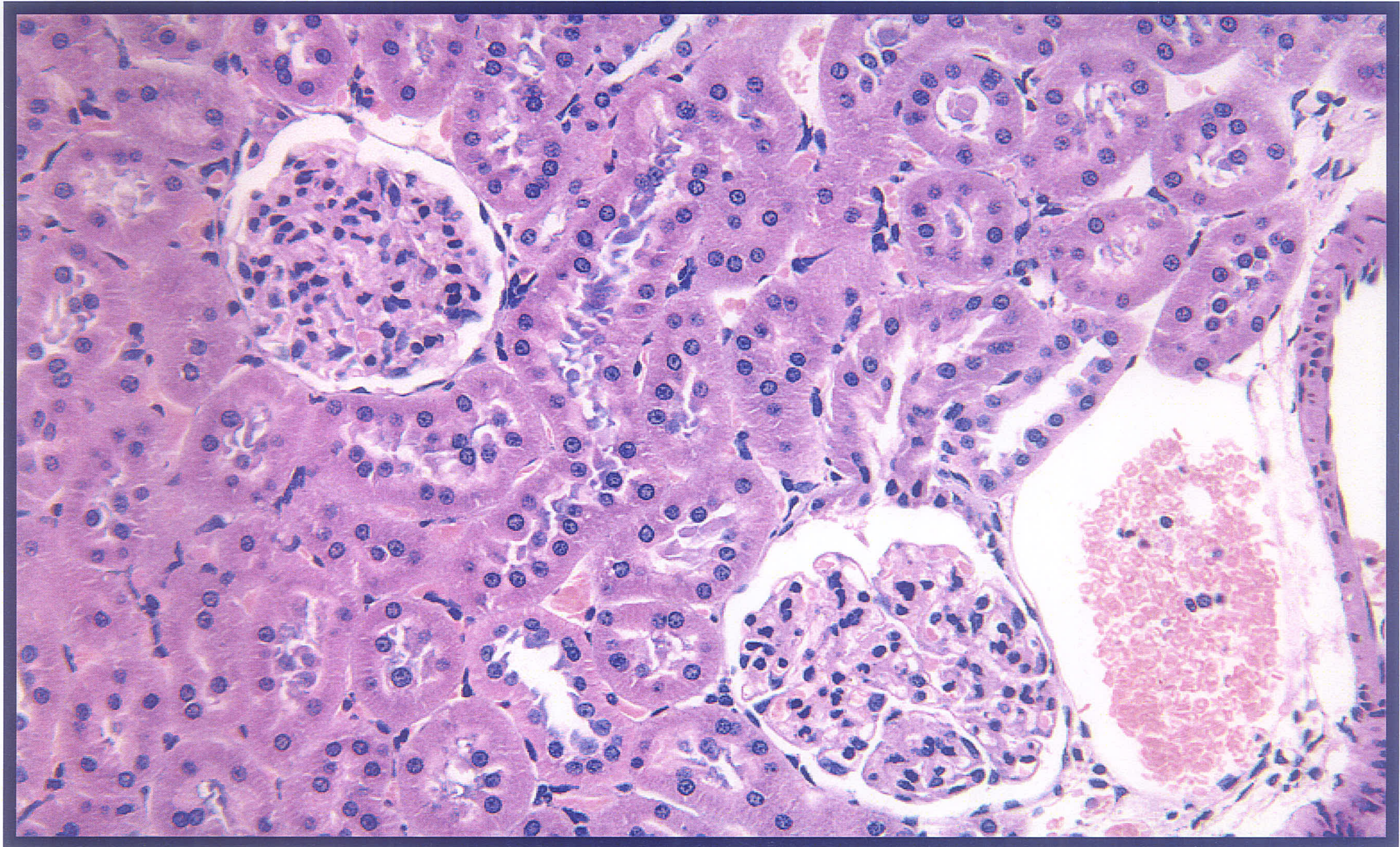

Figure 16 Kidney section from an obese *fa/fa* Zucker rat fed CLA diet for 8 weeks (20X magnification).

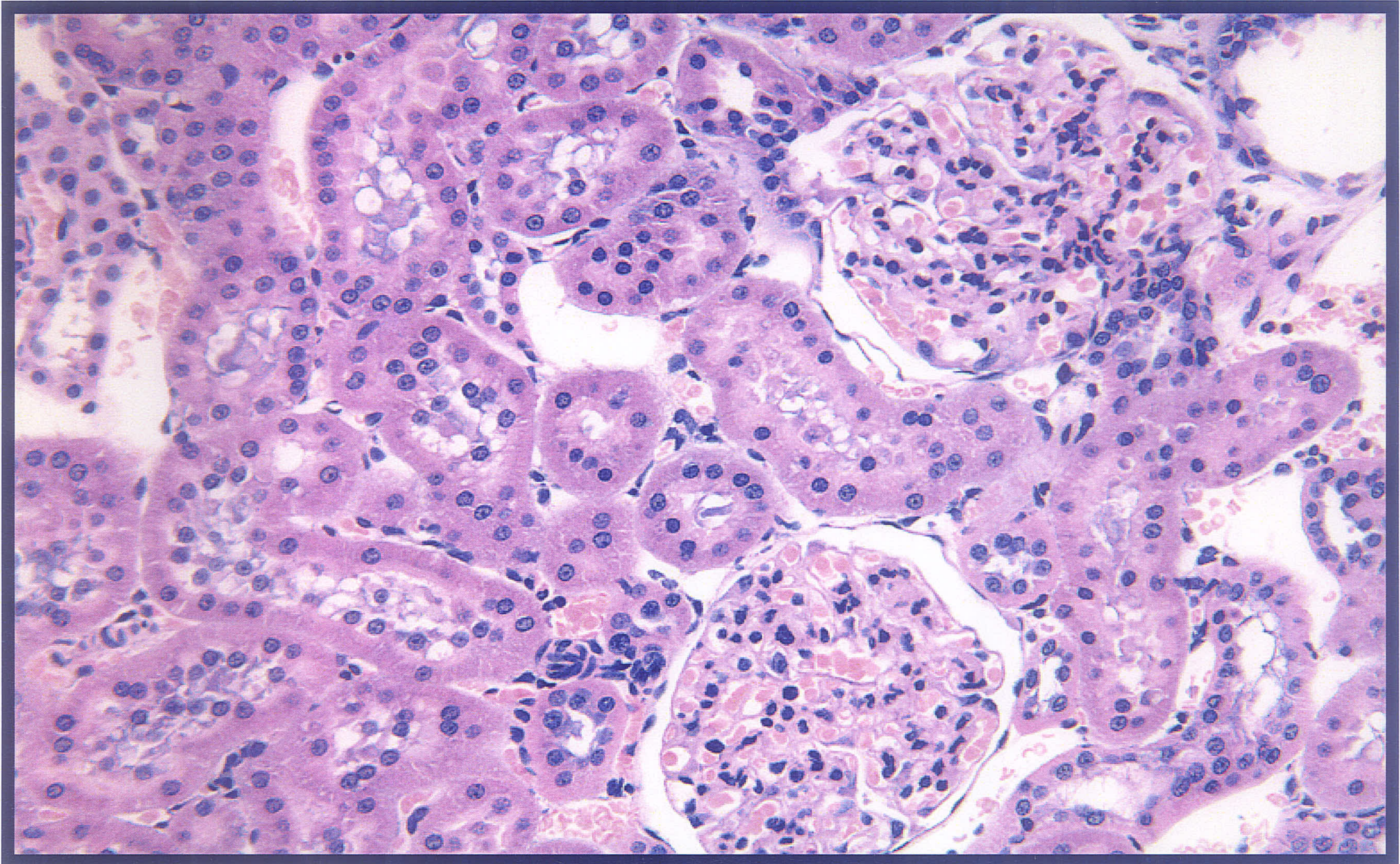

Figure 17 Kidney section from an obese *fa/fa* Zucker rat fed CTRL diet for 8 weeks (20X magnification).

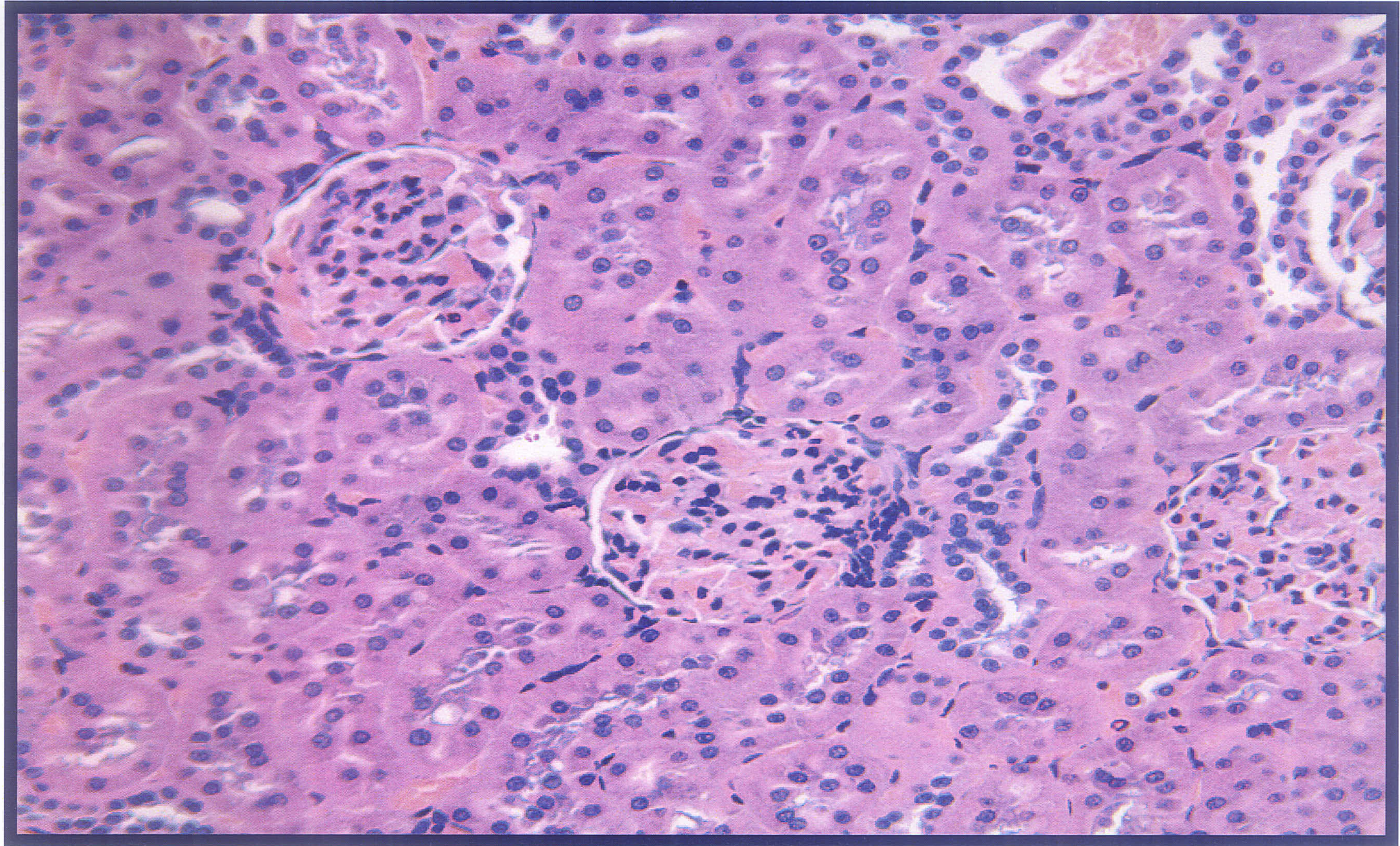

Figure 18 Kidney section from a lean Zucker rat fed CLA diet for 8 weeks (20X magnification).

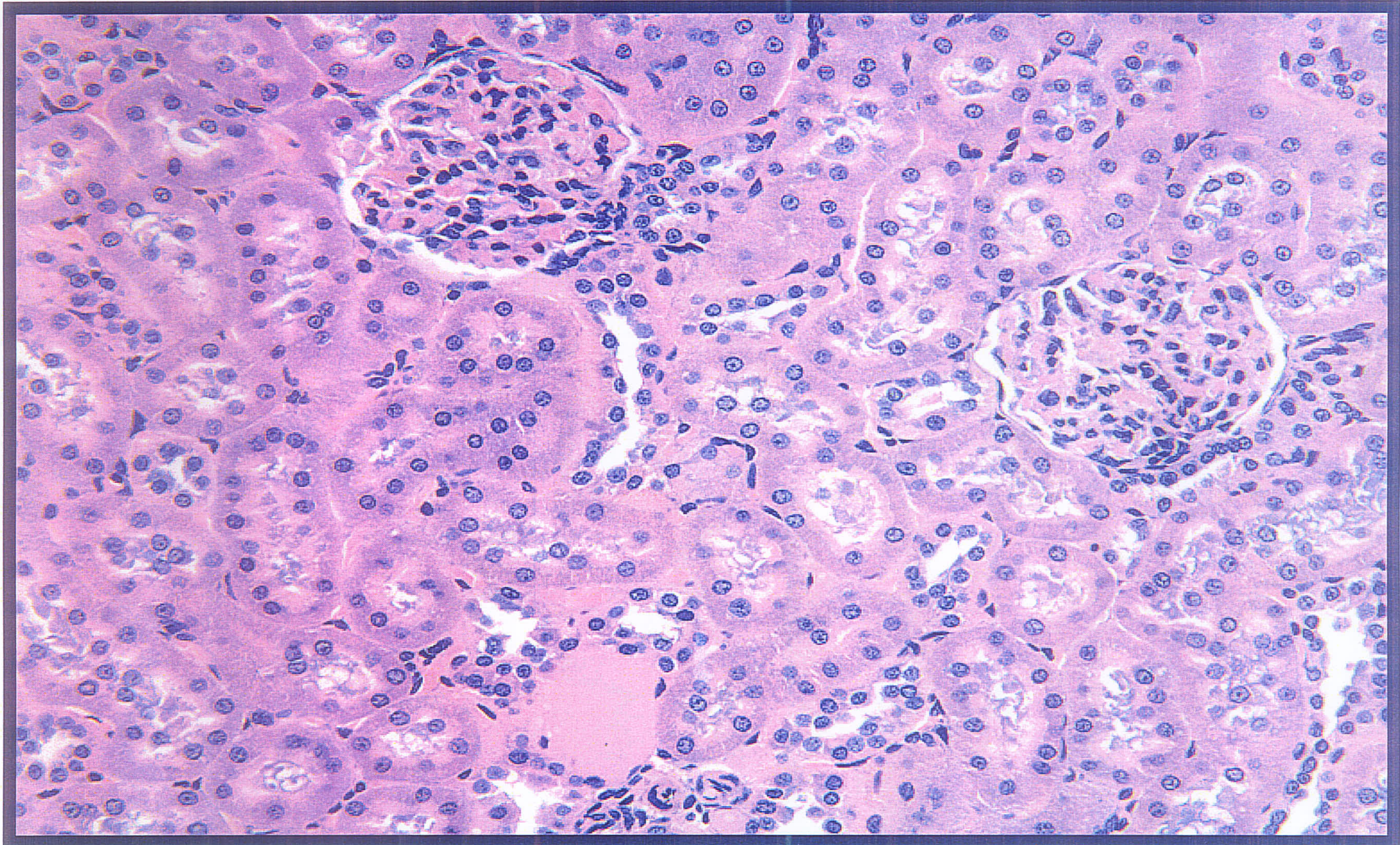

Figure 19 Kidney section from a lean Zucker rat fed CTRL diet for 8 weeks (20X magnification).

Table 9 Measurements of kidney function.

Measurement	faCLA	faCTRL	lnCLA	lnCTRL	Main Effects
Urine Volume (mL/12 hr)	6.46 ±0.40	7.98 ±0.73	5.40 ±0.75	5.52 ±0.59	Genotype $P < 0.0088$
Urine Creatinine (mg/12 hr)	3.18 ±0.25	3.80 ±0.27	4.88 ±0.29	5.23 ±0.31	Genotype $P < 0.0001$
Serum Creatinine (mg/dL)	0.922 ±0.057	0.933 ±0.093	0.591 ±0.021	0.603 ±0.022	Genotype $P < 0.0001$
Creatinine Clearance (mL/min)	0.494 ±0.053	0.613 ±0.068	1.87 ±0.28	1.34 ±0.25	Genotype $P < 0.001$
Urine Protein (mg/mg urine creatinine)	3.19 ±0.43	4.00 ±0.76	1.61 ±0.26	1.83 ±0.25	Genotype $P < 0.0004$

Data is presented as mean ± SEM (n = 10).
$P < 0.05$ is considered significantly different.

4.3.2 Urine Creatinine

Obese *fa/fa* Zucker rats had a 31% lower 12 hr urine creatinine excretion compared to lean Zucker rats (3.49 ± 0.19 mg/12 hr versus 5.05 ± 0.21 mg/12 hr, respectively, $P < 0.0001$).

4.3.3 Serum Creatinine

Obese *fa/fa* Zucker rats had a 55% higher serum creatinine concentration than lean Zucker rats (0.928 ± 0.053 mg/dL versus 0.597 ± 0.015 mg/dL, respectively, $P < 0.0001$).

4.3.4 Creatinine Clearance

Creatinine clearance can be used as a biological marker of kidney function as it is often used as an estimate of GFR (Maddox, Alavi, and Silbernick et al, 2002). Obese *fa/fa* Zucker rats had a 66% lower creatinine clearance compared to lean Zucker rats (0.55 ± 0.05 mL/min versus 1.61 ± 0.19 mL/min, respectively, $P < 0.001$).

4.3.5 Urine Protein

Obese *fa/fa* Zucker rats had a 109% higher 12 hr urine protein excretion compared to lean Zucker rats (3.60 ± 0.43 mg/mg urine creatinine versus 1.72 ± 0.18 mg/mg urine creatinine, respectively, $P < 0.0004$).

4.4 Protein Levels of Enzymes Involved in Eicosanoid Production in the Kidney

Table 10 summarizes $cPLA_2$, COX-1 and COX-2 protein levels in kidneys.

Table 10 $cPLA_2$, COX-1 and COX-2 protein levels in kidneys

Protein	faCLA	faCTRL	lnCLA	lnCTRL	Main Effects
Cytosol $cPLA_2$ (arbitrary units)	68.1 ±10.3 (n=8)	96.4 ±34.1 (n=7)	108.5 ±25.1 (n=8)	69.6 ±8.90 (n=8)	No effect
Particulate $cPLA_2$ (arbitrary units)	209.3 ±15.2	240.6 ±29.3	133.6 ±17.7	216.9 ±12.6 (n=6)	Diet P = 0.0256 Genotype P = 0.0181
Cytosol/Particulate $cPLA_2$ (arbitrary units)	0.317^{a} ±0.05 (n=8)	0.338^{a} ±0.07 (n=7)	0.838^{b} ±0.17 (n=8)	0.320^{a} ±0.05 (n=6)	Diet P = 0.0408 Genotype P = 0.0108 Interaction P = 0.0049
Particulate COX-1 (arbitrary untis)	160.8 ±32.0 (n=8)	140.0 ±23.6	128.3 ±33.9	140.6 ±22.7	No effect
Particulate COX-2 (arbitrary units)	264.7 ±52.3	390.2 ±52.9	97.5 ±10.1	130.3 ±43.2	Genotype P < 0.0001

Data is presented as mean ± SEM (n = 9, unless otherwise stated). Numbers in rows that contain different superscripts are significantly different at P < 0.05 using Tukey's post hoc test.

4.4.1 cPLA$_2$

There were no effects found for cytosol cPLA$_2$ protein levels. There were diet and genotype effects found for particulate cPLA$_2$ protein levels. CLA fed Zucker rats had 26% lower particulate cPLA$_2$ protein levels compared to CTRL fed Zucker rats (171.5 ± 14.6 arbitrary units versus 231.1 ± 18.1 arbitrary units, respectively, P = 0.0256). The obese *fa/fa* Zucker rats had 35% higher particulate cPLA$_2$ protein levels than lean Zucker rats (225.0 ± 16.4 arbitrary units versus 166.9 ± 15.8 arbitrary units, respectively, P=0.0181). In addition, there was a diet by genotype interaction found for the ratio of cytosol cPLA$_2$ to particulate cPLA$_2$ (P = 0.0049). The lean Zucker rats fed CLA had a 164%, 148% and 162% higher cytosol cPLA$_2$ to particulate cPLA$_2$ ratio than obese *fa/fa* Zucker rats fed CLA, obese *fa/fa* Zucker rats fed CTRL and lean Zucker rats fed CTRL, respectively, as shown by Tukey's post hoc analysis (0.838 ± 0.17 arbitrary units versus 0.317 ± 0.05 arbitrary units, 0.338 ± 0.07 arbitrary units and 0.320 ± 0.05 arbitrary units).

4.4.2 COX-1

There were no effects found for particulate COX-1.

4.4.3 COX-2

The obese *fa/fa* Zucker rats had 187% higher COX-2 protein levels than lean Zucker rats (327.4 ± 39.2 arbitrary units versus 113.9 ± 21.9 arbitrary units, respectively, P < 0.0001).

4.5 Protein Levels of PPARs in the Kidney

Table 11 summarizes PPAR α, PPAR β, and PPAR γ protein levels in kidneys.

4.5.1 PPAR α

There were diet and genotype effects found for particulate PPAR α protein expression. CLA fed Zucker rats had 21% lower PPAR α protein levels than those fed CTRL (589.7 ± 42.6 arbitrary units versus 742.9 ± 67.0 arbitrary units, respectively, P = 0.0490). Obese *fa/fa* Zucker rats had 22% lower PPAR α protein levels than the lean Zucker rats (582.7 ± 47.1 arbitrary units versus 748.0 ± 62.9 arbitrary units, respectively, P = 0.0244).

4.5.2 PPAR β

Obese *fa/fa* Zucker rats had 31% higher PPAR β protein levels than lean Zucker rats (196.1 ± 15.2 arbitrary units versus 149.3 ± 9.0 arbitrary units, respectively, P = 0.0113)

4.5.3 PPAR γ

There were no effects found for cytosol PPAR γ, particulate PPAR γ or the ratio of cytosol PPAR γ to particulate PPAR γ.

Table 11 PPAR α, PPAR β and PPAR γ protein levels in kidneys

Protein	faCLA	faCTRL	lnCLA	lnCTRL	Main Effects
Particulate PPAR α (arbitrary units)	562.8 ±61.8	602.5 ±74.3	616.7 ±61.1	883.3 ±92.9	Diet P = 0.0490 Genotype P = 0.0244
Particulate PPAR β (arbitrary units)	223.0 ±19.9	169.2 ±20.1	151.5 ±14.9	146.4 ±8.8 (n=7)	Genotype P = 0.0113
Cytosol PPAR γ (arbitrary units)	219.8 ±24.6	258.3 ±31.7	271.2 ±18.8 (n=8)	261.8 ±21.4	No effect
Particulate PPAR γ (arbitrary units)	38.46 ±3.44 (n=6)	36.32 ±2.84 (n=8)	42.14 ±4.74 (n=8)	37.75 ±3.03 (n=7)	No effect
Cytosol/Particulate PPAR γ (arbitrary units)	5.62 ±0.74 (n=6)	7.11 ±1.25 (n=8)	7.47 ±0.73 (n=7)	6.10 ±0.50 (n=6)	No effect

Data is presented as mean ± SEM (n = 9, unless otherwise stated)

5 DISCUSSION

DN is the number one cause of ESRD in Canada and the Western world, accounting for approximately 30% of all patients who have ESRD (Meltzer et al, 1998; Gall et al, 1993; Gall et al, 1991). Approximately 50% of patients who have ESRD associated with diabetes have Type 2 diabetes (Ibrahim et al, 1997; Gall et al, 1993; Gall et al, 1991).

Low protein diets, vegetable based protein diets and n-3 fats have shown beneficial effects in the attenuation of various renal diseases (Ogborn et al, 2003 – in press; Ogborn et al, 2002; Aukema et al, 2001; Ogborn et al, 1999; Anderson et al, 1998; Yanagisawa et al, 1998; Clark et al, 1995; Ingram et al, 1995; Tomobe et al, 1994; Yanagisawa et al, 1994; Aukema et al, 1992; Walker et al, 1989; Zatz et al, 1985), however, little research has focused on dietary intervention in the Type 2 diabetes patient with DN

Recent, investigations using CLA have shown that this naturally occurring fat is able to alter the production of eicosanoids and rate-limiting enzymes involved in eicosanoid production in a rat model of Polycystic Kidney Disease (unpublished data from Dr. Aukema's lab; Ogborn et al, 2003 – in press). In addition, CLA has been shown to slow the progression of this form of renal disease (Ogborn et al, 2003 - in press). Therefore, CLA may ameliorate Polycystic Kidney Disease via alterations in eicosanoid production. Also newly discovered is that the renal production of eicosanoids is associated with the progression of early DN in a rat model of Type 2 diabetes and that Type 2 diabetes patients have significantly higher urinary levels of these eicosanoids

compared to healthy individuals (Okumura et al, 2003; Hishinuma et al, 2001; Okumura et al, 2000).

PPARs are newly discovered, present in the kidney and have potential role(s) for the attenuation of DN (Guan et al, 2001; Guan and Breyer, 2001; Nicholas et al, 2001; Asano et al, 2000; Yang et al, 1999; Guan 1997; Braissant 1996). CLA has proven to be a ligand for PPAR α and PPAR γ (Moya-Camarena et al, 1999; Houseknecht et al, 1998). PPAR α regulates genes involved in lipid transport and lipid oxidation in the liver as well as anti-inflammation (Yurkova et al, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Devchand et al, 1996; Schoonjans et al, 1996). The lipid lowering effects of fibrates, also ligands for PPARs, are thought to be due to the activation of PPAR α (Ouali et al, 1998; Fruchart et al, 1999; Forman et al, 1997; Kliewer et al, 1997). Reduction of hyperlipidemia in the obese *fa/fa* Zucker rat with the use of fibrates has been shown to reduce proteinuria and glomerular injury (Kasiske et al, 1988).

PPAR γ is a key transcription factor involved in the terminal differentiation of adipocytes and regulates genes involved in anti-inflammation and glucose homeostasis (Houseknecht et al, 2002; Picard and Auwerz, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Gregoiré et al, 1998; Schoonjans et al, 1996). Ligands for PPAR γ, such as the insulin sensitzers TZDs, have been shown to ameliorate DN in a rat model of Type 1 and Type 2 diabetes as well as in humans with Type 2 diabetes (McCarthy et al, 2000; Imano et al, 1998; Fujii et al, 1997; Lehmann et al, 1995).

The **hypotheses** of this study were that: 1) early CLA feeding to obese *fa/fa* Zucker rats, an animal model of obesity and the Type 2 diabetes "pre-diabetic" state

would prevent deterioration in renal function and delay glomerular enlargement after 8 weeks of feeding and 2) CLA would alter steady-state protein levels of rate-limiting enzymes involved in eicosanoid synthesis as well as eicosanoids themselves and alter the protein levels of PPARs in kidney tissue.

To test these hypotheses, the **objectives** of this research were to analyze the kidneys of 6 week old obese *fa/fa* Zucker rats and lean Zucker rats fed either 1.5% CLA by weight or a CTRL diet for 8 weeks for the following:

1. early histological change in the size of glomeruli via hemotoxylin and eosin staining of kidney cross-sections with subsequent measurement of MGV
2. steady-state renal levels of COX-1, COX-2, and $cPLA_2$ protein using Western Immunoblotting
3. *ex vivo* renal production of PGE_2, PGI_2, and TXA_2 using a competitive binding protein assay
4. steady-state renal levels of PPAR α, PPAR β, and PPAR γ protein using Western Immunoblotting

5.1 Animal Growth

5.1.1 Feed Intake

Due to a *fa* mutation in the leptin receptor, obese *fa/fa* Zucker rats are unable to suppress appetite, and therefore, are hyperphagic (Phillips et al, 1999; Upton et al, 1998; Bray, 1977; Zucker, 1965). In this study, obese *fa/fa* Zucker rats ate more than lean Zucker rats throughout the 8-week study period. Analysis of total feed intake revealed the obese *fa/fa* Zucker rats ate 43% more food than lean Zucker rats. This translates into

obese *fa/fa* Zucker rats having eaten approximately 8 grams more diet per day compared to lean Zucker rats. These results are consistent with results obtained by Ionescu et al in 1985 who also found that during the first 13 to 14 weeks of age obese *fa/fa* Zucker rats were eating approximately 8 grams more diet per day than lean Zucker rats. These results are also comparable to Sisk et al's (2001) results in which obese *fa/fa* Zucker rats ate 7 g more food per day than lean Zucker rats. There was no overall effect of dietary CLA on food intake in obese *fa/fa* Zucker or lean Zucker rats. These results are similar to Houseknecht et al's results from 1998 who also found that there was no overall effect of feeding 1.5% dietary CLA for 2 weeks on food intake in fatty rats; however, Houseknecht et al used the ZDF rat and did not investigate the effect of CLA feeding in the lean Zucker rat. In addition, Houseknecht et al (1998) used a dietary CLA mixture that was 90% pure with the major isomers being 42% *cis*-9, *trans*-11 CLA and 43.5% *trans*-9, *cis*-11 CLA, unlike the current study in which the dietary CLA was 86.3% pure with the major isomers being 29.89% *trans*-10, *cis*-12 CLA, 28.63% *cis*-9, *trans*-11 CLA, 17.75% *cis*-11, *trans*-13 CLA and 15.54% *trans*-8, *cis*-10 CLA. Results of this study are also similar to Sisk et al's results in 2001, which also concluded there was no effect of dietary CLA on food intake in obese *fa/fa* Zucker rats; however, CLA was fed at a lower level (0.5%) for a shorter time period (5 weeks). The dietary CLA mixture in the Sisk et al (2001) study was 97% pure with the major isomers being 42.6% *cis*-9, *trans*-11 CLA and 45.6% *trans*-10, *cis*-CLA.

5.1.2 Body Weight

As their name implies, the obese *fa/fa* Zucker rat is an obese rat and therefore, a genotype effect would be expected between the obese *fa/fa* Zucker rat and the lean Zucker rat, as is the case in this study. At the end of the study, obese *fa/fa* Zucker rats were 58% heavier than lean Zucker rats. These results are consistent with the results obtained by Upton et al in 1998 who also found that obese *fa/fa* Zucker rats gained approximately 57% more weight than lean Zucker rats (fed standard lab chow). Similar findings were reported by Kasiske et al in 1985, who found obese *fa/fa* Zucker rats were 52% heavier than lean Zucker rats at 14 weeks of age (fed standard lab chow).

Another reason why one would expect the obese *fa/fa* Zucker rat to weigh more than lean Zucker rats is because they ate 43% more food. However, it is interesting to note that pair-feeding of obese *fa/fa* Zucker rats to the food intake of lean animals does not lead to the normalization of body fat content or the elimination of the characteristic defects of these animals, as reviewed by Bray in 1977. In addition, as reported by Sisk et al in 2001, 0.5% dietary CLA reduced adiposity in lean but not obese *fa/fa* Zucker rats. The efficiency of weight gain was not affected by diet in the Sisk et al (2001) study, but the obese *fa/fa* Zucker rats were more efficient in their gain to feed ratio than lean Zucker rats. Therefore, obese *fa/fa* Zucker rats may weigh more in this study because they ate more than lean Zucker rats, but also because they may be more efficient in converting feed to weight gain. In this study, the weight gain to total feed ratio was larger in the obese *fa/fa* Zucker rats compared to the lean Zucker rats (0.25 ± 0.003 versus 0.20 ± 0.004, respectively, $P < 0.0001$). There was no effect of dietary CLA on the weight gain to total feed ratio.

5.2 Disease Progression

5.2.1 Kidney Weight

After 8 weeks, obese *fa/fa* Zucker rats fed CLA had an 11% smaller kidney weight compared to those fed CTRL. Conclusions regarding whether feeding CLA decreased kidney weight or prevented an increase in kidney weight cannot be drawn because kidney weight was not measured at an earlier time point. However, because these animals are in the early stage of development one would expect kidney weight to be smaller during the early stage of life. If kidney weight was measured in obese *fa/fa* Zucker rats after the first week of CLA and CTRL feeding one could have taken the ratio of obese *fa/fa* Zucker kidney weight in the CLA fed animals versus those fed CTRL to determine if CLA had decreased kidney weight or prevented an increase. Obese *fa/fa* Zucker rats fed CLA had a 14% larger kidney weight than lean Zucker rats fed CLA, while obese *fa/fa* Zucker rats fed the CTRL diet had a 28% larger kidney weight than lean Zucker rats fed CTRL. Kasiske et al in 1985 found that at 14 weeks of age, obese *fa/fa* Zucker rats, fed standard lab chow, had a 24% higher kidney weight than lean Zucker rats. This percentage closely resembles the 28% larger kidney weight seen in the obese *fa/fa* Zucker rats fed the CTRL diet versus the lean Zucker rats fed the CTRL diet, thus further supporting the results of this study which show a beneficial effect of dietary CLA on disease progression. However, one would expect obese *fa/fa* Zucker rats to have larger kidney weights based on the fact they are larger animals.

When final kidney weight was expressed per 100 grams of final body weight, there was a genotype effect observed such that obese *fa/fa* Zucker rats had a 23% lower kidney to body weight ratio compared to lean Zucker rats. This too is consistent with

findings by Kasiske et al in 1985 who found that obese *fa/fa* Zucker rats had a 21% lower kidney to body weight ratio compared to lean Zucker rats. However, because final kidney weight of obese *fa/fa* Zucker rats fed CLA and CTRL was only 14% and 28% heavier than lean Zucker rats fed CLA and CTRL, respectively, but obese *fa/fa* Zucker rats were 58% heavier than lean Zucker rats, one would expect that the ratio of final kidney weight to final body weight would be smaller for the obese *fa/fa* Zucker rats compared to the lean Zucker rats.

5.2.2 Mean Glomerular Volume

Feeding CLA had a beneficial effect on disease progression in the obese *fa/fa* Zucker rat, based on MGV. Obese *fa/fa* Zucker rats fed CLA for 8 weeks had a 28% smaller MGV compared to those fed the CTRL diet. Conclusions regarding whether feeding CLA decreased MGV or prevented an increase in MGV cannot be drawn as MGV was not measured at any earlier time point. However, because these animals are in the early stage of development, one would expect MGV to be smaller during the early stage of life. If MGV was measured in obese *fa/fa* Zucker rats after the first week of CLA and CTRL feeding one could have taken the ratio of obese *fa/fa* Zucker MGV in the CLA fed animals versus those fed CTRL to determine if CLA had decreased MGV or prevented an increase. Obese *fa/fa* Zucker rats had a 30% and 84% larger MGV than lean Zucker rats fed CLA and CTRL, respectively, at 14 weeks of age. It has been reported in the literature that obese *fa/fa* Zucker rats fed standard lab chow have significant glomerular hypertrophy at 14 weeks of age compared to lean Zucker rats, as determined by glomerular area measurements (Coimbra et al, 2000; Kasiske et al, 1985).

When glomerular area is converted to glomerular volume using the equation used in this study, the obese *fa/fa* Zucker rats in the Coimbra et al (2000) study had a glomerular volume that was 31% larger than lean Zucker rats while the obese *fa/fa* Zucker rats in the Kasiske et al (1985) study had a glomerular volume that was 46% larger than the lean Zucker rats. These percentages are slightly higher than the percentage seen in this study for obese *fa/fa* Zucker rats fed CLA versus lean Zucker rats fed CLA, thus reflecting the beneficial effect of dietary CLA on disease progression.

In 2002, Maddox, Alavi, and Santelia et al measured MGV in obese *fa/fa* Zucker rats and lean Zucker rats (fed standard lab chow) using a technique very similar to the one used in this study. The researchers found that at 60 weeks of age (the end of the study period), obese *fa/fa* Zucker rats had a 90% larger MGV compared to lean Zucker rats. In a study by Maddox, Alavi, and Silbernick et al in 2002, which investigated the effects of soy protein on renal disease progression in obese *fa/fa* Zucker rats, it was found that at 24 weeks of age, obese *fa/fa* Zucker rats (fed casein and soy) had a 61% larger MGV than lean Zucker rats (there was no diet effect). Both Maddox, Alavi, and Santelia et al (2002) and Maddox, Alavi, and Silbernick et al (2002) used female Zucker rats, while Coimbra et al (2000) and Kasiske et al (1985) used males. Estrogen levels in female obese *fa/fa* Zucker rats may promote accelerated renal injury as investigated by Gades et al in 1998. Hence, one would expect renal damage in female obese *fa/fa* Zucker rats to occur earlier than male obese *fa/fa* Zucker rats.

5.2.3 Creatinine Clearance

Renal function was worse in obese *fa/fa* Zucker rats compared to lean Zucker rats as indicated by a 66% lower creatinine clearance in the obese *fa/fa* Zucker. These results are not consistent with results obtained by Kasiske et al in 1985 who found that there were no differences in GFR between obese *fa/fa* Zucker rats and lean Zucker rats at 14 week of age, however, by 28 weeks of age there was a significant difference with obese *fa/fa* Zucker rats having a 73% lower GFR than lean Zucker rats. Kasiske et al (1985) used inulin clearance as a measure of GFR while this study used creatinine clearance. Therefore, differences in GFR measurements may provide some explanation as to why a lower GFR was found earlier in this study. As reported by Namnum et al in 1983, creatinine clearance is not the best reliable marker of GFR in the rat because net creatinine transport across the renal tubule is bidirectional and is influenced by the serum creatinine level and in this study serum creatinine was different in obese *fa/fa* Zucker rats versus lean Zucker rats.

In relation to the equation used to calculate creatinine clearance, obese *fa/fa* Zucker rats had a 32% higher 12 hr urine volume, a 31% lower 12 hr urine creatinine excretion, and a 55% higher serum creatinine concentration than lean Zucker rats. Therefore, the 66% lower creatinine clearance observed in obese *fa/fa* Zucker rats compared to lean Zucker rats is a result of the 31% lower 12 hr urine creatinine excretion and 55% higher serum creatinine concentration in the obese *fa/fa* Zucker.

5.2.4 Urine Protein

Obese *fa/fa* Zucker rats had 109% higher 12 hr urinary protein excretion than lean Zucker rats, which is another indicator of poor kidney function. In 1985, Kasiske et al found that urine albumin excretion was significantly greater for obese *fa/fa* Zucker rats compared to lean Zucker rats by 14 weeks of age. Maddox, Alavi, and Santelia et al, in 2002, found that urinary protein was significantly increased between 6 and 26 weeks of age in female obese *fa/fa* Zucker rats compared to lean Zucker rats.

5.3 Protein Levels of Enzymes Involved in Eicosanoid Production in the Kidney

5.3.1 $cPLA_2$

The $cPLA_2$ enzyme is responsible for the release of AA from the sn-2 position of membrane phospholipids (Smith and Murphy 2002; Wilton and Waite, 2002). The $cPLA_2$ enzyme is a ubiquitously distributed enzyme that is found in the cytosol, and once activated by an increase in cytosolic Ca^{2+}, it translocates to the membrane surface (Wilton and Waite 2002; Evans et al, 2001; Hirabayashi et al, 2000; Bonventre, 1999; Hirabayashi et al, 1999; Gronich et al, 1988). Therefore, inactive $cPLA_2$ will be found in the cytosol homogenate of kidney fractions, while activated $cPLA_2$ will be found in the particulate homogenate of kidney fractions.

Zucker rats fed CLA had 26% lower particulate $cPLA_2$ protein levels compared to Zucker rats fed CTRL. In addition, obese *fa/fa* Zucker rats had 35% higher particulate $cPLA_2$ protein levels than lean Zucker rats. This suggests that obese *fa/fa* Zucker rats have greater protein levels of the active form of $cPLA_2$ and that feeding obese *fa/fa* Zucker rats CLA results in lower protein levels of the active form of $cPLA_2$ compared to

those fed CTRL. Greater protein levels of the active form of $cPLA_2$ in the obese *fa/fa* Zucker rat may mean that renal disease in this animal model may be related to inhanced AA release from membrane phospholipids, and therefore, greater production of TXA_2. Hence, renal disease in the obese *fa/fa* Zucker rat may occur via a mechanism similar to the OLETF rat (rat model of Type 2 diabetes) in which TXA_2 levels are increased beginning at 14 weeks of age and is maintained at a high level as nephropathy progresses (Okumura et al, 2000). The protein level of $cPLA_2$ is known to be increased in the rat and mouse models of Polycystic Kidney Disease and feeding CLA reduces an increase in $cPLA_2$ protein levels (unpublished data Dr. Aukema; Aukema et al, 2002). Feeding CLA to the rat model of Polycystic Kidney Disease has been shown to attenuate disease progression in this form of renal disease, as seen by the inhibition of inflammation and subsequent fibrosis (Ogborn et al, 2003 – in press)

Lean Zucker rats fed CLA had a higher cytosol $cPLA_2$ to particulate $cPLA_2$ ratio than any other experimental group. This suggests that lean Zucker rats fed CLA had lower protein levels of the activated form of $cPLA_2$ compared to lean Zucker rats fed CTRL and obese *fa/fa* Zucker rats fed CLA or CTRL.

Taking into consideration the results above, it looks as if CLA may be decreasing or preventing an increase in the protein levels of the activated form of $cPLA_2$ in the Zucker rat. However, a lower $cPLA_2$ protein level does not necessarily infer less $cPLA_2$ activity. In addition, because obese *fa/fa* Zucker rats have higher protein levels of the active form of $cPLA_2$, it does not necessarily infer greater protein activity. Measuring AA release would have given an indication of $cPLA_2$ activity.

5.3.2 COX-2

Obese *fa/fa* Zucker rats had 187% higher COX-2 protein levels compared to lean Zucker rats. In this case, if downstream metabolites of COX-2 activity had been assessed, such as PGE_2, PGI_2 and TXA_2 formation, one would be able to draw more concrete conclusions regarding the role of COX-2 in eicosanoid production in the obese *fa/fa* Zucker rat.

It is interesting to note that particulate $cPLA_2$ protein levels and COX-2 protein levels are both higher in the obese *fa/fa* Zucker rat than the lean Zucker rat; the $cPLA_2$ enzyme being responsible for AA release and the inducible COX-2 enzyme being responsible for the production of PGI_2, PGE_2 and TXA_2 from AA. It has been documented in the literature that AA released by $cPLA_2$ is supplied predominately to COX-2 in the delayed response, in which a small amount of AA is released gradually, whereas a burst release of AA is supplied to COX-1 (Murakami et al, 2000; Murakami et al, 1999). In 2000, Okumura et al discovered that in the OLETF rat, an animal model of Type 2 diabetes, TXA_2 and PGI_2 (as measured by their urinary metabolites TXB_2 and 6-keto-$PGF_{1\alpha}$, respectively) were significantly higher than normal rats beginning at 14 weeks of age and as they got older TXA_2 levels peaked and remained steady and PGI_2 levels decreased to that of normal rats, while renal disease progressed. In addition, it has been found that the ratio of TXA_2 to PGI_2 (as measured by their urinary metabolies11-dehydro-thromboxane B_2 and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, respectively) in patients with Type 2 diabetes is significantly higher than that of healthy individuals due to a higher TXA_2 and lower PGI_2 (Hishinuma et al, 2001). It could be possible then that in this study the $cPLA_2$ supplies AA to COX-2 over time (i.e. in a delayed response) in the

obese *fa/fa* Zucker rat, thus playing a role in renal disease progression as indicated by the obese *fa/fa* Zucker rats having larger MGVs, larger kidney weights, greater urinary protein excretions and lower creatinine clearances compared to the lean Zucker rats. Based on evidence from this study and evidence from Okumura et al (2000) and Hishinuma et al (2001), COX-2 may be responsible for TXA_2 production and COX-1 may be responsible for PGI_2 production in the Type 2 diabetes metabolic state.

5.4 Protein Levels of PPARs in the Kidney

5.4.1 PPAR α

Zucker rats fed CLA had 21% lower PPAR α protein levels than Zucker rats fed CTRL. PPAR α regulates the expression of genes in the liver that are involved in lipid transport and lipid oxidation as well as anti-inflammation (Yurkova et al, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Devchand et al, 1996; Schoonjans et al, 1996). CLA is a known ligand for PPAR α in rat hepatoma cells (Moya-Camarena et al, 1999). In 1998, Houseknecht et al demonstrated that feeding dietary CLA for 2 weeks lowered circulating free fatty acids in 8 week old Zucker diabetic fatty (*fa/fa*) rats. The researchers hypothesized that PPAR α activation may be involved as the lipid lowering effects of fibrates are thought to be due to PPAR α ligand binding (Fruchart et al, 1999; Ouali et al, 1998; Kliewer et al, 1997; Forman et al, 1997). Reduction of hyperlipidemia in the obese *fa/fa* Zucker rat with the use of fibrates has been shown to reduce glomerular injury (Kasiske et al, 1988). Reductions in plasma lipids in the obese *fa/fa* Zucker rat may be related to favorable changes in the fatty acid content of the kidney and therefore favorable changes in renal eicosanoid production

(Kasiske et al, 1991; Wheeler et al, 1991; Clark et al, 1990). Therefore, in this study, lower PPAR α protein levels in CLA fed Zucker rats may have unfavorable effects on ameliorating renal disease progression. However, it is not known if low PPAR α protein levels are beneficial in the amelioration of DN. It is interesting to note that PPAR α has been shown to down-regulate the expression of COX-2 in cell culture (Staels et al, 1998). In 2000, Broeders et al reported that some fibrates induce renal dysfunction in humans with underlying renal insufficiency or who are taking multiple other medications, as seen by increases in blood urea and creatinine. Broeders et al (2000) speculated that the down-regulation of COX-2 expression via fibrate ligand binding to PPAR α may inhibit the production of vasodilatory prostaglandins within the kidney which can lead to impaired renal function. If this is the case, CLA lowering of PPAR α protein levels in this study may be beneficial. However, the pathophysiology of fibrate-induced increases in blood urea and creatinine remains to be fully elucidated.

Obese *fa/fa* Zucker rats had 22% lower PPAR α protein levels than lean Zucker rats at 14 weeks of age. As stated above, one of the key characteristics of the obese *fa/fa* Zucker rat is hyperlipidemia (Phillips et al, 1999; Upton et al, 1998; Kasiske et al, 1992; McCaleb and Sredy, 1992; Ionescu et al, 1985). Because PPAR α plays a central role in lipid metabolism and ligands of PPAR α lowers hyperlipidemia, it may be speculated that hyperlipidemia in obese *fa/fa* Zucker rats may be related to lower PPAR α protein levels (Yurkova et al, 2002; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Fruchart et al, 1999; Ouali et al, 1998; Kliewer et al, 1997; Forman et al, 1997; Devchand et al, 1996; Schoonjans et al, 1996). In addition, as assessed by Coimbra et al (2000), at 14 weeks of age, glomerular and tubulointerstitium monocyte/macrophage

infiltration is at its peak in obese *fa/fa* Zucker rats. Therefore, it may be speculated that this insult may be related to the low PPAR α protein levels observed in the obese *fa/fa* Zucker rats of this study. Again, it is not known if low PPAR α protein expression is beneficial in the amelioration of DN.

5.4.2 PPAR β

Obese *fa/fa* Zucker rats had 31% higher PPAR β protein levels than lean Zucker rats. PPAR β is highly expressed in the kidney; however, its specific function is unknown. It has been suggested as having an important role in cell survival (Wu, 2000). In addition, PPAR β agonists have been shown to have favorable effects on blood lipid profiles in insulin resistant db/db mice as reflected by increased high density lipoproteins (Leibowitz et al, 2000). Therefore, as DN progresses in the obese *fa/fa* Zucker rat, alterations in kidney tissue and function may stimulate higher PPAR β protein expression to aid in the survival of the kidney or its ensuing death as well as alter lipid metabolism.

5.5 Possible Effects of CLA on Renal Disease Progression

5.5.1 Enzymes Involved in Eicosanoid Production

In 2000, Okumura et al discovered that in the OLETF rat, an animal model of Type 2 diabetes, TXA_2 and PGI_2 (as measured by their urinary metabolites TXB_2 and 6-keto-$PGF_{1\alpha}$, respectively) were significantly higher than normal rats beginning at 14 weeks of age and as they got older TXA_2 levels peaked and remained steady and PGI_2 levels decreased to that of normal rats, while renal disease progressed. In addition, it has been found that the ratio of TXA_2 to PGI_2 (as measured by their urinary metabolites 11-

dehydro-thromboxane B_2 and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, respectively) in patients with Type 2 diabetes is significantly higher than that of healthy individuals due to a higher TXA_2 and lower PGI_2 (Hishinuma et al, 2001). Thus, high TXA_2 levels and low PGI_2 levels may play a pivotal role in DN progression in the Type 2 diabetes metabolic state.

TXA_2 is a potent vasoconstrictor and platelet aggregator, while PGI_2 is potent vasodilator and platelet anti-aggregator (Fitzgerald et al, 1987; Needleman et al, 1986). Analogs of TXA_2 and PGI_2 alter the expression of genes encoding basement membrane proteins in vitro in differentiated mouse tetrocarcinoma cells and human glomerular mesangial cells (Bruggeman et al, 1993; Bruggeman et al, 1991). TXA_2 analogs increase the steady-state mRNA levels for all three laminin chains (A, B1, B2), type IV collagen and fibronectin and decrease the level of mRNA for heparin sulfate proteoglycan, while PGI_2 has the opposite effect. As stated in section 2.5, thickening of glomerular and tubular basement membranes is characteristic of DN progression. In 1990, Ledbetter et al concluded that basement membrane thickening in a Type 2 diabetes mouse model is partly a result of an unbalanced increase in the production of type IV collagen and that inhibition of TXA_2 prevented an increase in type IV collagen mRNA.

TXA_2 and PGI_2 are formed in the COX pathway from its metabolic precursor AA. The $cPLA_2$ enzyme is an important rate-limiting enzymes involved in this process. Recent unpublished data from Dr. Aukema's lab has revealed that in the rat model of Polycystic Kidney Disease CLA alters protein levels of $cPLA_2$ in the kidney. In addition, CLA has been shown to slow the progression of this form of renal disease (Ogborn et al, 2003 - in press). Therefore, CLA may ameliorate Polycystic Kidney Disease via

alterations in eicosanoid production. In this study, Zucker rats fed CLA had reduced particulate $cPLA_2$ protein levels compared to those fed CTRL. Obese *fa/fa* Zucker rats fed CLA had smaller kidney weights and MGVs compared to those fed the CTRL diet. Therefore, it could be possible that CLA is decreasing or preventing an increase in $cPLA_2$ protein levels in the Zucker rat kidney, thus decreasing AA release, and therefore, decreasing the production of the "2" series of eicosanoids which in turn aides in the amelioration of renal disease progression.

5.5.2 PPARs

PPAR α regulates the expression of genes in the liver that are involved in lipid transport and lipid oxidation in as well as anti-inflammation (unpublished data Dr. Taylor; Corton and Anderson, 2000; Escher and Wahli, 2000; Keller et al, 2000; Devchand et al, 1996; Schoonjans et al, 1996). CLA is a known ligand for PPAR α in rat hepatoma cells (Moya-Camarena et al, 1999). In 1998, Houseknecht et al demonstrated that feeding dietary CLA for 2 weeks lowered circulating free fatty acids in 8 week old Zucker diabetic fatty (*fa/fa*) rats. The researchers hypothesized that PPAR α activation may be involved via CLA ligand binding as the lipid lowering effects of fibrates, also a PPAR ligand, are thought to be due to PPAR α activation (Fruchart et al, 1999; Ouali et al, 1998; Forman et al, 1997; Kliewer et al, 1997). Hypolipidemic drugs, such as fibrates, reduce hyperlipidemia in the obese *fa/fa* Zucker rat and after approximately 32 weeks of treatment, significantly reduce proteinuria, mesangial expansion and glomerularsclerosis (Kasiske et al, 1988). Reduction in plasma lipids in the obese *fa/fa* Zucker rat may be related to favorable changes in the fatty acid content of the kidney and, therefore,

favorable changes in renal eicosanoid production (Kasiske et al, 1991; Wheeler et al, 1991; Clark et al, 1990). Therefore, the role of PPARs and eicosanoids in DN may be related. Yurkova et al (2002) revealed that after 8 weeks of feeding dietary CLA, there was no dietary effect of CLA on serum triglyceride levels in obese *fa/fa* Zucker rats at 14 weeks of age, however, expression of genes involved in lipid transport and metabolism in the liver were elevated 1.3-1.5 fold in obese *fa/fa* Zucker rats fed CLA. Although CLA reduced PPAR α protein levels in the kidney in this study, it cannot be concluded that PPAR α activity in the kidney was reduced as this was not measured. Indeed, it seems that CLA has inhibited or decreased PPAR α protein levels in the kidney in this study, and therefore, may have unfavourable effects in the amelioration of DN; however, it is not known if low levels of PPAR α protein is beneficial in the amelioration of DN and in fact, there is some speculation that ligand binding of fibrates to PPAR α may have unfavourable effects on eicosanoid production in the kidney (Broeders et al, 2000). Therefore, the lowering effect of CLA on PPAR α protein levels may be beneficial.

5.6 Summary

Feeding CLA to obese *fa/fa* Zucker rats for 8 weeks resulted in smaller MGVs and kidney weights compared to those fed CTRL. Amelioration of these pathological signs of renal disease in this model of obesity and the "pre-diabetic" state may be related to lower particulate $cPLA_2$ protein levels observed in the kidneys of Zucker rats fed CLA compared to those fed CTRL. Feeding CLA to Zucker rats lowered PPAR α protein levels, which may or may not have aided in the amelioration of DN.

Research in unveiling the mechanisms involved in CLA action is relatively new, which poses many challenges in itself, in addition to the fact that different CLA isomers may have different mechanisms of action in different tissues. However, this research thesis is the first, to the writer's knowledge, to investigate the effect of dietary CLA on DN in a rat model of obesity and the "pre-diabetic" state and suggests that dietary CLA may have beneficial effects in ameliorating DN in the very early stages of Type 2 diabetes development.

5.7 Limitations and Strengths

A limitation of the study is that all parameters investigated were done so at only one time point; the end of the study. As a consequence, conclusions regarding whether changes observed were due to a decrease in a specific measurement as opposed to the inhibition of an increase could not be stated.

Another limitation of this study is that measuring steady-state levels of a protein does not give insight into protein activity. Measuring downstream metabolites of the protein of interest would have provided more insight into the protein's role in renal disease progression. The fact that problems arose with the analysis of *ex vivo* COX enzyme activity and eicosanoid production is related to this limitation.

Another limitation of the study is that the investigator was not blinded to the rat group assignment and therefore, this may have inadvertently cast bias on data collection.

Upon completion of this study, it was calculated that the power to detect diet effects between experimental groups was: 1) 97% for the measurement of MGV, 2) approximately 44% for the measurement of PPAR protein levels (based on a randomly

selected PPAR data set), 3) approximately 36% for the measurement of enzyme protein levels (based on a randomly selected enzyme data set), and 4) approximately 26% for the measurement of parameters of kidney function (based on a randomly selected kidney furntion data set). Power was calculated using the variance of the data set, an n = 10 (except for an n = 9 for the PPAR and enzyme data), and a Type I error of 0.05. Therefore, the power to detect diet effects for MGV was strong and, therefore, the sample size was adequate; however, an n = 23 to 30 would have been required to provide a power of at least 80% for the detection of a diet effect in the PPAR, enzyme and kidney function data. As a consequence, sample size was a limitation

The fact that dietary CLA mixture used in this study was composed of various isomers is another limitation of this study. The dietary effects observed in this study cannot be attributed to a specific CLA isomer. In addition, because other researchers use CLA mixtures of various isomer compositions, it is difficult to compare the results of this study with that found in the literature.

A strength of this study is the animal model used. The male obese *fa/fa* Zucker rat is an excellent model of obesity and the Type 2 diabetes "pre-diabetic" state as it very closely resembles human obesity and impaired glucose tolerance, which can eventually lead to Type 2 diabetes mellitus and renal complications associated with this disease. Investigations involving dietary intervention during the "pre-diabetic" state may aid in early amelioration of renal disease progression and perhaps even the prevention of renal disease.

Another strength of this study is that, to the writer's knowledge, this research thesis is the first to investigate the effect of dietary CLA on DN in a rat model of obesity and the Type 2 diabetes "pre-diabetic" state.

5.8 Directions for Future Research

To further elucidate the underlying cause(s) of renal disease in the obese *fa/fa* Zucker rat and how CLA may affect renal disease progression, the following parameters of investigation would be beneficial:

- measure $cPLA_2$, COX-1 and COX-2 enzyme activity
- measure *ex vivo* kidney production of eicosanoids using specific $cPLA_2$, COX-1, and COX-2 inhibitors
- investigate the hemodynamic and morphological effects of eicosanoids on renal function and structure
- investigate the specific role(s) of PPARs on renal function and structure
- feed specific CLA isomers and measure *ex vivo* kidney production of eicosanoids as well as the renal expression of PPAR responsive genes
- feed differing amounts of CLA isomers for differing lengths of time and measure *ex vivo* kidney production of eicosanoids as well as the renal expression of PPAR responsive genes.

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