DIETARY SOY PROTEIN AND RENAL DISEASE SELECTIVELY ALTER CYCLOOXYGENASE ISOFORMS AND PROSTANOID PRODUCTION IN HAN:SPRD-*cy* RATS

BY

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

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ABSTRACT

Dietary soy protein feeding initiated at weaning slows the progression of renal injury and inflammation in the Han:SPRD-*cy* rat model of autosomal dominant polycystic kidney disease (ADPKD). However, the effects of initiating dietary soy protein prior to weaning have not been studied. Cyclooxygenase (COX) isoforms are altered in the kidneys of these rats. Therefore, the objectives of this study were two fold: 1) to determine the effects of initiating dietary soy protein prior to weaning, and 2) to examine the effects of disease and dietary soy protein on renal prostanoid production and COX isoforms. Han:SPRD-*cy* rats were given casein or soy protein in the maternal and/or post-weaning diets in a 2×2 design. Dietary soy protein in the post-weaning diet reduced cyst growth, inflammation and cell proliferation, as previously reported. Dietary soy protein in the maternal diet also independently reduced renal inflammation by 22% and lowered proteinuria by 33%. Soy protein in either the maternal or post-weaning diet reduced renal cell proliferation, with either intervention being equally effective. Soy protein in the post-weaning diet resulted in lower levels of particulate $cPLA_2$, but did not affect COX isoform levels; soy protein in the maternal diet did not alter the levels of $cPLA_2$ or COX isoforms. Therefore, the effects of disease and soy protein on prostanoids and COX isoform activities were examined in rats given soy in the post-weaning diet. The presence of disease resulted in higher kidney levels of TXA_2, PGE_2, PGI_2 and COX isoform activities. The effect of disease on the relative changes in the levels of prostanoids was in the order of $TXA_2 > PGI_2 > PGE_2$. Soy protein in the post-weaning diet lowered the *in vitro* steady-state production of TXA_2 by 49%, and resulted in less TXA_2 and PGI_2 produced by COX-2 activity by 47% and 36%, and less PGI_2 produced by COX-1

activity by 76% in diseased kidneys. Therefore, dietary soy protein in the maternal diet provides further benefits in offspring with renal disease. Beneficial effects of dietary soy protein in the post-weaning diet are associated with select effects on prostanoid production in polycystic kidneys.

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This journal is full of challenges, but I feel so fortunate to have a chance to learn so much and to experience what I have experienced.

LIST OF ABBREVIATIONS

AA	arachidonic acid
APS	ammonium persulfate
ADPKD	autosomal dominant polycystic kidney disease
BUO	bilateral ureteral obstruction
CHF	congestive heart failure
CLA	conjugated linoleic acid
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
EGF	epidermal growth factor
EDTA	ethylene-diamine-tetraacetic acid
EGTA	ethylene glycol-bis (β-amino ethyle ether) N,N,N',N'-tetracetic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ESRD	end-stage renal disease
GFR	glomerular filtration rate
NOS	nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
NO	nitric oxide
OD	optical density
PGE_2	prostaglandin E_2
PGI_2	prostaglandin I_2
PKD	polycystic kidney disease

PLA_2	phospholipase A_2
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulphate
SUN	serum urea nitrogen
TBS	tris base solution
TEMED	tetramethylethylenediame
TXA_2	thromboxane A_2

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1. LITERATURE REVIEW AND PRELIMINARY FINDINGS

1.1 Autosomal Dominant Polycystic Kidney Disease (ADPKD)

1.1.1 Description

Polycystic kidney disease (PKD) is one of the most frequent disorders among inherited renal diseases and it affects more people than the combined incidences of cystic fibrosis, muscular dystrophy, hemophilia, Down's syndrome, and sickle cell anemia [1]. Autosomal dominant polycystic kidney disease (ADPKD) is the most common form of PKD, affecting 600,000 US citizens and an estimated 12.5 million people worldwide [1,2]. ADPKD accounts for 8-10% of the cases of end-stage renal disease (ESRD) and costs for dialysis and transplantation exceed more than $1.5 billion annually in the United States [1,3,4]. Therefore, ADPKD is a serious medical, social and economic problem for patients and their families as well as for society as a whole.

ADPKD is characterized by abnormal growth and development of renal cysts, interstitial fibrosis and interstitial inflammation [5]. The enlarged kidneys in ADPKD are due to the presence of multiple cysts which are derived from all segments of the nephron, and are distributed throughout the cortex and medulla, resulting in a loss of reniform structure of the kidney and disappearance of the cortico-medullary boundary [6]. Cysts vary in size from a few millimetres to several centimetres, and the cyst fluids contained within may vary in colour and composition from clear and yellow to turbid and brown, the latter reflecting hemorrhage into the cyst cavity [6].

There are at least three components contributing to renal cyst formation, including epithelial cell hyperproliferation, fluid accumulation within the cysts, and extracellular matrix abnormalities [5,6,7,8]. Renal tubular walls are lined by a single layer of epithelial

cells, and a precisely controlled balance between cellular proliferation and programmed cell death (apoptosis) is essential for normal growth and differentiation of the kidney and maintenance of normal renal structure after birth [9]. The proliferation of normal renal tubular epithelial cells ceases before birth; however, in ADPKD, apoptosis is abnormally persistent and can destroy much of the normal renal parenchyma, thereby allowing cystic epithelia to proliferate [9]. Fluid accumulation is required for the development and growth of cysts to prevent collapse of the cyst walls. The sequestration of fluid in cysts is due to accumulation of glomerular filtrate and/or transepithelial secretion of fluid [3,8]. Mislocalization of the sodium pump (Na^+/K^+-ATPase) is implicated as the possible mechanism of fluid accumulation in ADPKD [8]. Expanding cysts must remodel their physical environment as they enlarge; therefore, thickened basement membranes and alterations in extracellular matrix composition also play important roles in cystogenesis [3,4,5,7,8]. Fibroblasts underlying the renal cystic tubular portions are involved in the over expression of matrix compounds. Immunoreactivity for type IV collagen, laminin, and fibronectin has been shown to be increased in basement membranes and extracellular matrix [5,7,8]. These alterations cause marked functional disturbances. For example, epithelia from ADPKD kidneys are more adherent to matrixes made up of collagen type I or IV than are normal epithelia and such defects may impair the cell movements required for morphogenesis of the kidney [9].

Other than cyst formation and interstitial fibrosis, ADPKD kidneys also exhibit significantly widened interstitial spaces with prominent inflammatory infiltrates, consisting mainly of lymphocytes and macrophages [5]. The presence of these interstitial inflammatory cells also suggests that substances released by them, such as cytokines and

lymphokines, may play a role in the development of interstitial fibrosis since cytokines are shown to be associated with tissue modeling [5,10]. Taken together, renal cysts and interstitial fibrosis are associated with renal dysfunction, and renal inflammation may contribute to interstitial fibrosis formation. However, how renal cysts and fibrosis are formed, and whether or not cysts trigger interstitial inflammation and further develop interstitial fibrosis still needs further investigation.

In 85% of patients, the gene responsible for the disease is *PKD1*, whereas in 5 to 10% of patients, the mutation is in the *PKD2* gene [11]. A very small percentage of ADPKD patients have no mutations in *PKD1* or *PKD2*, suggesting that there may be a third form of the disease, although the proposed gene – *PKD3* – has not been identified yet [4,9,11]. *PKD1* is on the short arm of human chromosome 16p, *PKD2* is on chromosome 4q, and the genomic location of the proposed *PKD3* is still unknown [4]. The *PKD1* protein, polycystin 1, has 4,302 amino acids and a molecular weight of 460 kDa; the *PKD2* protein, polycystin 2, has 968 amino acids and a molecular weight of 110 kDa [4,12]. Polycystin 1 is a membrane receptor capable of binding and interacting with many proteins, carbohydrates, and lipids and eliciting intracellular responses through phosphorylation pathways, whereas polycystin 2 is a calcium-permeable channel [9]. A wide range of mutations, such as deletions, insertions, and point mutations in *PKD1* or *PKD2* can cause ADPKD; however, the exact pathogenic mechanism of cyst formation is still unclear. It is agreed by most scientists that polycystin 1 and polycystin 2 may interact with each other and also with a variety of other proteins, and that they may be involved in signal transduction pathways that lead to the activation of a plethora of genes necessary for renal tubular development and maintenance [13].

ADPKD is not only a renal disease; it is a systemic disorder with a myriad of manifestations. Other than renal cysts, interstitial fibrosis and interstitial inflammation, renal manifestations also include hypertension, renal concentrating defect, impaired endocrine functions, urinary tract infection, acute and chronic pain, nephrolithiasis, hematuria, and renal failure; gastrointestinal manifestations include hepatic cysts, pancreatic cysts, and colonic diverticula; cardiovascular manifestations include intracranial aneurysms and cardiac valvular abnormalities [1,2,4,14,15]. Hypertension is very commonly seen in ADPKD patients and it has been suggested that the alteration of intrarenal circulation in renal ischemia areas caused by the cysts is associated with the activation of the renin-angiotensin-aldosterone system [14]. More than 60% of ADPKD patients develop kidney failure or end-stage renal disease (ESRD), and hypertension is one of the most important risk factors for the progression of renal insufficiency as well as a contributor to overall cardiovascular mortality [2,6,14]. The risk of cardiovascular mortality of kidney failure patients treated with dialysis is about 500-fold (age 25-35) to 5-fold (age > 85) higher than that of the general population [16].

1.1.2 Treatments for Autosomal Dominant Polycystic Kidney Disease

To date, there is still no cure for ADPKD; however, with the increased understanding of the molecular genetic and cellular pathophysiologic mechanisms responsible for the development of ADPKD, several interventions have been used to delay the disease progression. Methylprednisolone, an anti-inflammatory drug with antifibrogenic effects, was shown to diminish the progression of the cystic change, decrease the extent of interstitial inflammation and fibrosis in both Han:SPRD-*cy* rat and DBA/2FG-*pcy/pcy*

mouse models of PKD [17]. Cilazapril, an angiotensin converting enzyme inhibitor, was shown to reduce renal cyst volume and lower blood pressure in Han:SPRD-*cy* rat model of ADPKD [18].

1.2 Dietary Intervention in Renal Disease

1.2.1 Dietary Fat Intervention

Several different types of dietary interventions have been tested in animal models of ADPKD. Both the level and type of dietary fat have been studied. Reducing the level of dietary fat retards disease progression in both the CD1-*pcy/pcy* mouse and the Han:SPRD-*cy* rat model of ADPKD [19,20]. One study showed that male CD1-*pcy/pcy* mice fed a high fat (20% soybean oil) diet for 130 days compared to a low fat (4% soybean oil) diet had higher kidney weight relative to body weight and higher kidney weight relative to liver weight, suggesting the potential detrimental effects of high dietary fat [19]. In male Han:SPRD-*cy* rats with kidney disease, consuming a high fat diet (20% soybean oil) compared to a low fat diet (5% soybean oil) for 6 weeks resulted in increased kidney weights, kidney water content, cyst score, serum urea and serum creatinine, indicating greater kidney disease progression and worsened renal function [20]. These studies demonstrated that a low fat diet compared with a high fat diet slows disease progression in animal models of ADPKD.

Different lipid sources, such as flaxseed, fish oil and conjugated linoleic acid (CLA), were studied in animal models of ADPKD as well. Flaxseed, a rich source of α-linolenic acid (n-3), has been reported to ameliorate the chronic interstitial nephritis associated with the renal cystic disease in Han:SPRD-*cy* rats [21]. In this study, flaxseed-fed

animals had lower serum creatinine, less cystic change, less renal fibrosis, and less macrophage infiltration of the renal interstitium than controls, while apoptosis and proliferation of renal tubular epithelial cells were not altered. The mechanism responsible for the beneficial effects was hypothesized to be through competition with linoleic acid for Δ6-desaturase and a reduction in arachidonic acid (AA) synthesis.

Fish oil, which also contains high amounts of n-3 fatty acids, marginally slows early cyst formation in DBA/2FG-*pcy/pcy* mice in one study [22], but in other studies, no beneficial effect was found when fish oil was introduced during both the growing and adult stages in *pcy* mice [19,23]. Long-term feeding of dietary fish oil did not improve survival in *pcy* mice [24]. However, in the Han:SPRD-*cy* rats with renal disease, the detrimental effects of a high fat diet in early renal injury are ameliorated by fish oil [25]. Moveroer, 8 weeks of CLA feeding to the Han:SPRD-*cy* rats with renal disease significantly reduced renal inflammation and renal fibrosis [26,27].

1.2.2 Dietary Protein Intervention

Early studies revealed that ingestion of protein aggravates the clinical manifestations of patients with renal insufficiency [28], and dietary protein restriction modifies the course of progressive renal insufficiency. In several rat models of renal disease, such as partially nephrectomized rats, diabetic rats and spontaneously hypertensive rats, dietary protein restriction can modify hemodynamic abnormalities and prevent progressive renal damage [29]. However, studies of dietary protein restriction in other animal models, such as partially nephrectomized dogs, yielded conflicting results [29].

As to the effects of dietary protein restriction in human renal disease, the results from previous studies have not been consistent. In one study, 149 patients with various renal diseases were fed either a low-protein diet (0.4 – 0.6g protein/kg body weight/day) or a control diet (55 – 70g protein/day) for at least 18 months [30]. Patients on the low-protein diet showed decreases in serum urea and phosphate concentrations and in the 24 h excretion of urea, phosphate, and protein. The slope of reciprocal serum creatinine over time was calculated, and the steepness of the slope was used to estimate the rate of progression of renal insufficiency. Results showed that the slope of reciprocal creatinine over time was three to five times lower in patients on the low-protein diet than on the control diet. Therefore, the authors concluded that dietary protein restriction is an acceptable and effective way of delaying renal functional deterioration. The beneficial effects of dietary protein restriction in chronic renal patients were also reported in many other human studies [29]. However, there are also some studies which have not demonstrated a protective effect of dietary protein restriction in renal patients. In one study, patients with late chronic renal insufficiency fed a low-protein diet (0.6g protein/kg body weight/day) for up to two years did not have lower disease progression compared to patients on the control diet (1g protein/kg body weight/day) [31]. Another study done by the Modification of Diet in Renal Disease (MDRD) study group reported that among patients with moderate renal insufficiency, the slower decline in renal function that started four months after the introduction of the low-protein diet suggested a small benefit of this dietary intervention. Among patients with more severe renal insufficiency, a very-low-protein diet, as compared with a low-protein diet, did not significantly slow the progression of renal disease [32]. However, shortly after this study

was published, numerous secondary analyses of this MDRD study were undertaken to clarify the effects of protein restriction on the rate of decline in glomerular filtration rate (GFR), urine protein excretion, and the onset of end-stage renal disease [33]. Prescribed protein intake and achieved protein intake were calculated and compared, and the rate of progression of renal disease was correlated with achieved total protein intake. The authors concluded that even though these secondary results can not be regarded as definitive, the balance of evidence is more consistent with the hypothesis of a beneficial effect of protein restriction than with the contrary hypothesis of no beneficial effects.

The differing results in these studies may be due to several factors, such as different types of renal diseases, differences in study designs, sample size, the length of experiment period, methods used to assess renal disease progression and the absence of compliance data. Several studies used meta-analysis to better examine the effects of dietary protein restriction on renal function and disease progression in previous published studies [34,35,36]. One study included a total of 1413 patients from five studies of non-diabetic renal diseases (mean length of follow-up, 18 to 36 months) and 108 patients from five studies of type I diabetes mellitus (mean length of follow-up, 9 to 35 months). Results showed that dietary protein restriction slows the progression of both diabetic and non-diabetic renal diseases [34]. Another study pooled the results of 13 randomized controlled trials including 1919 patients (mean length of follow up was 21.8 months), and found that although dietary protein restriction retards the rate of decline in GFR among patients with renal disease, the magnitude of the effect was relatively small [35]. However, the authors also suggested that it is possible that studies of longer duration could show a greater effect of a low protein diet. In 2000, another study used meta-

analysis to determine the efficacy of low protein diets in preventing the progression of chronic renal failure towards ESRD and therefore delaying the need for starting maintenance dialysis [36]. A total of 1494 patients suffering from moderate to severe renal failure were analyzed. 753 patients received reduced protein intake and 741 patients received a high protein intake for at least one year. Results showed that reducing protein intake in patients with chronic renal failure reduces the occurrence of ESRD by 40% as compared with unrestricted protein intake. However, in all of these human studies, dietary protein restriction was initiated at a stage when renal filtration capacity was already reduced to 25-50% of normal and it seems that the benefits of dietary interventions in the later stages of disease might be marginal.

In 2003, one study was done to examine the association between total protein intake and renal function decline over an 11-year period in women with normal renal function or mild renal insufficiency [37]. 1624 women were enrolled in this study and the results showed that high protein intake was not associated with renal function decline in women with normal renal function, but it may accelerate renal function decline in women with mild renal insufficiency.

So far, no studies have been undertaken to determine the effects of early dietary protein restriction in human ADPKD, but in 1992, the first study was done to investigate the effects of early dietary interventions in the animal model of ADPKD [23]. The interventions were started before the presentation of clinical symptoms and the abnormal measurements of renal function. In sub-study 1, weanling DBA/2FG-*pcy/pcy* mice (30 days of age) were fed either a normal (25%) or low (6%) casein diet with 10% of either sunflower seed oil or fish oil for 120 days in a 2 × 2 design. In mice on a normal protein

diet, kidney weight relative to body weight was higher, and kidney phospholipid to kidney weight was lower compared to the mice on low protein diet. The results indicated that the increased kidney size was largely due to increased cyst development. In sub-study 2, weanling DBA/2FG-*pcy/pcy* mice were fed either a normal (25%) or low (6%) casein diet with corn oil as the lipid source for 120 days. Morphometric analysis showed that percentage cyst area and total cyst area were lower in kidney sections derived from mice on the low-protein diet compared to normal protein diet. The authors concluded that early dietary protein restriction in ADPKD prior to clinical manifestation of symptoms of the disease may have a significant impact on the pathogenesis of ADPKD. In addition, different dietary lipid types in this study did not show effects on disease progression of ADPKD.

In 1994, another study was done to further examine the effects of early dietary protein restriction on disease progression and survival in the DBA/2FG-*pcy/pcy* mouse model of ADPKD [38]. These mice were slightly older (70 days of age) but were still in the very early stages of the disease and exhibited no apparent clinical manifestations of disease. In sub-study 1, mice were fed either a normal protein (25% casein) or a low-protein (6% casein) diet for 105 days. Kidney weight relative to body weight, kidney water contents, and total and percentage cyst area were lower, while relative renal phospholipid and triglyceride contents were higher in mice fed the low-protein diet compared with the normal protein diet. In sub-study 2, mice were fed either a normal protein (25% casein) or a low-protein (6% casein) diet. Results showed that the mean lifespan for DBA/2FG-*pcy/pcy* mice on the low-protein diet was 24% longer than that for those mice on the normal protein diet. This study demonstrated that early dietary protein reduction initiated

before the manifestation of clinical symptoms of disease slows disease progression and lengthens survival in DBA/2FG-*pcy/pcy* mouse model of ADPKD.

Early dietary protein restriction was also introduced to the Han:SPRD-*cy* rat model of ADPKD [39]. At 60 days of age, heterozygous Han:SPRD-*cy* rats were put on either a normal protein (20% casein) or a low-protein (8% casein) diet for 4 months. Results showed that dietary restriction was associated with the reduction of mean body weight, mean renal volume adjusted for body weight, total cyst volume, and mean serum creatinine and urea levels. Therefore, early dietary protein restriction was shown to be a potent method for modifying the course of ADPKD in the Han:SPRD-*cy* rat model as well.

Even though these animal studies demonstrated that early dietary protein restriction can slow down disease progression in animal models of ADPKD, whether similar results would occur in human ADPKD patients still needs further investigation. Patient compliance is a crucial factor when dietary protein restriction is prescribed in humans [31,32,33]. In addition to poor compliance, protein restriction early in life could increase the risk of malnutrition or affect normal growth. Therefore, manipulation of the type of protein in the diet has been considered for potential effects on renal disease progression.

1.2.3 Dietary Soy Protein Intervention

Soy protein has been recognized as having potential roles in the prevention and treatment of chronic diseases, such as cancer, heart disease and kidney disease [40]. With respect to kidney disease, subtotally nephrectomized Wistar rats ingesting 24% soy protein-based diets for 10-13 weeks were shown to have improved survival rate, reduced

proteinuria, renal hypertrophy and renal histological damage compared with rats ingesting 24% casein diet [41,42]. Long-term feeding of dietary soy protein compared with casein increases life span and decreases renal pathology in Fischer 344 rats [43,44]. BKS.cg-m +Lepr^{db}/+Lepr^{db} (db/db) mice, which are commonly used as a model of type II diabete mellitus and diabetic nephropathy, were shown to have improved glomerular macromolecular permeability and slower development of diabetic nephropathy after consuming a soy protein diet for 21-26 weeks [45]. Moreover, in rats with chronic nephritic syndrome, soy protein feeding improved renal function and reduced renal damage and proinflammatory cytokines [46,47].

With respect to ADPKD, in one study, weanling Han:SPRD-*cy*/+ rats received either 20% soy protein or 20% casein based diets for 8 weeks [48]. Soy-fed rats had lower serum creatinine, lower urinary ammonium excretion, reduced renal cysts, renal fibrosis, macrophage infiltration, renal tubular cell proliferation and apoptosis. This study demonstrated that early dietary soy protein intervention reduces both tubular and interstitial pathology in the Han:SPRD-*cy* rat model of ADPKD.

Another study was done to examine the effects of dietary soy protein on the progression of cyst growth in 60-day-old male DBA/2FG-*pcy/pcy* mouse model of ADPKD [49]. In addition, the effects of genistein, an isoflavonoid present in soy protein, on cyst growth were also examined. In sub-study 1, mice were fed either casein (15g protein/100g diet) or soy protein (15g protein/100g diet) diet for 90 days. Total kidney weight, kidney weight relative to body weight, kidney water content, mean cyst volume, and plasma urea were reduced in mice fed soy protein diet. But no differences were found between these two groups with respect to final body weight, plasma creatinine and

plasma total protein content. In sub-study 2, mice were fed casein (15g protein/100g diet) diet with or without genistein (0.05g protein/100g diet) for 60 days. Genistein supplementation did not reduce total kidney weight, kidney weight relative to body weight, and the renal enlargement and cyst development associated with progression of ADPKD. The authors concluded that soy protein is effective in retarding cyst development in the DBA/2FG-*pcy/pcy* mice and this beneficial effect may not be related to its genistein content.

The effects of different dietary protein levels and different protein sources on early disease progression were also examined in both male and female CD1-*pcy/pcy* mouse model of ADPKD [50]. 10-week-old CD1-*pcy/pcy* mice were fed either soy protein diet or casein diet at a level of 6 % or 17.4 % for 13 weeks. With respect to protein sources, animals fed soy protein diet had lower relative kidney weights, lower cyst scores, less kidney water content and serum urea nitrogen (SUN) compared to animals fed a casein diet. With respect to protein levels, dietary protein reduction resulted in lower relative kidney weights, lower cyst scores, less kidney water content and SUN. The results showed that both dietary protein source and level significantly affect CD1-*pcy/pcy* mice, with the effects of dietary soy protein being most pronounced in female mice fed the low protein diet and the effects of protein reduction being most pronounced in mice fed soy protein diet.

A recent study also demonstrated that dietary soy protein compared with casein delays disease progression in the very early stage of renal disease [51]. Soy protein feeding resulted in reduced cyst growth and renal fibrosis as early as 1-3 weeks after it

was fed to weaning Han:SPRD-*cy* rats with renal disease, showing the beneficial effects of soy protein in the early stage of the disease.

These studies have demonstrated that both early dietary protein restriction and early dietary soy protein intervention retard renal disease progression in both *pcy* mice and Han:SPRD-*cy* rats with renal disease. However, the exact mechanisms are not clear. The kidney is a rich source of eicosanoids which regulate renal hemodynamics, water and solute transport, and renin secretion [52]. Altered eicosanoid production has been shown in several diseased animal models [53,54]. In diseased kidneys, the increased eicosanoid production seems to be involved in maintaining renal hemodynamics and inflammatory processes in response to and as part of the renal injury [54,55,56]. It is known that soy protein compared to meat protein has a reduced effect on renal hyperfiltration and proteinuria. These effects may be explained by lowered plasma glucagon and renal vasodilatory prostaglandin secretion [58]. However, the effects of dietary soy protein on eicosanoid production in ADPKD are not known.

1.3 Eicosanoids

1.3.1 Eicosanoids and Eicosanoid Biosynthetic Enzymes in the Kidney

The major precursor of eicosanoids is the C_{20} polyunsaturated fatty acid, AA [59]. The pathways leading to the eicosanoids are known collectively as the 'arachidonate cascade'. There are three major pathways within the cascade, including the cyclooxygenase (COX), lipoxygenase, and cytochrome P-450 (CYP450) pathways [56,59,60]. Products formed via the COX pathway are called prostanoids including prostaglandins and thromboxanes. The numerical subscripts are used to indicate the

number of double bonds. The prostanoids with '2' subscript are derived from AA; the '1' series prostanoids are from dihomo-γ-linolenic acid, and the '3' series prostanoids are from eicosapentaenoic acid. The lipoxygenase pathway leads to the formation of mono-, di, and trihydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs), and lipoxins (LXs). Epoxyeicosatrienoic acids (EETs) are formed via the cytochrome P-450 pathway [56,59]. While all three pathways are present in the kidney, the COX pathway is the major pathway for AA metabolism [56] and is the pathway focused on in this thesis.

Prostanoids formed in the COX pathway occur in three stages: (1) release of AA from membrane phospholipids by phospholipase A_2 (PLA_2), (2) conversion of AA to prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by COX, (3) conversion of PGH_2 to prostaglandins and thromboxanes by prostanoid-specific enzymes [59,60].

Prostanoids act as short-range messengers, affecting tissues near the cells that produce them. They are not stored by cells, but are synthesized and released rapidly in response to hormones [59]. In the resting state, free AA is not present in significant amounts in tissues; it is esterified in membrane phospholipids at the *sn*-2 position [60,61]. When hormonal stimuli, such as angiotensin II or thrombin, interact with cell surface receptors, PLA_2 is activated. The release of AA by PLA_2 is believed to be the rate-limiting step for the biosynthesis of prostanoids. Mammalian cells have several types of PLA_2, including cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$), and Ca^{2+}-independent PLA_2 ($iPLA_2$). $cPLA_2$ with molecular weight of 85 kDa, is the only PLA_2 enzyme that shows significant selectivity toward phospholipids bearing AA at the *sn*-2 position [62]. Although α-linolenic acid and eicosapentaenoic acid (EPA) in the phospholipids are substrates for $cPLA_2$ as well, their low quantities in natural membranes make AA the

major substrate for $cPLA_2$ under physiological conditions [61,62]. $cPLA_2$ is expressed in most adult human tissues including kidney and in many cell types including macrophages, neutrophils, platelets, endothelial cells, vascular smooth muscle cells and renal mesangial cells [61]. $cPLA_2$ is present mainly in the cytosol under resting conditions and its activation is controlled by cytoplasmic Ca^{2+} levels and phosphorylation. When a cell-surface receptor is activated by the stimuli, it transduces a signal into the cell. An increase in cytoplasmic Ca^{2+} levels causes $cPLA_2$ to translocate from cytosol to the perinuclear region including the nuclear envelope and endoplasmic reticulum (ER), and to release AA from the membrane phospholipids [59,61,62].

Once AA is released, it can be acted upon by COX to form prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2). COX is a bifunctional enzyme exhibiting both cyclooxygenase and peroxidase activities. The cyclooxygenase component introduces molecular oxygen to convert AA into PGG_2, and the peroxidase component converts PGG_2 into PGH_2, the precursor of prostaglandins and thromboxanes [59,60]. Two isoforms of the cyclooxygenase enzyme, COX-1 and COX-2, have been identified. Both isoforms have a molecular weight of 71 kDa, and are almost identical in length, with just over 600 amino acids, of which 63% are in an identical sequence [63]. COX-1 was thought to be constitutively expressed in nearly all cell types at a constant level while COX-2 was thought to be inducible and normally absent from cells [63,64]. However, recent studies have shown that COX-1 could also be upregulated in inflammation and COX-2 is constitutively expressed in a number of non-inflammatory tissues, such as kidney and brain [64,65,66,67]. In rat kidneys, COX-1 expression is found in glomerular mesangial cells, distal convoluted tubule, connecting tubule, cortical and medullary

collecting ducts, and COX-2 expression is found in glomeruli, cortical thick ascending limb (cTAL), macula densa and medullary interstitial cells [68]. Within cells, COX-1 and COX-2 are found on the luminal surfaces of the ER and on the inner and outer membranes of the nuclear membrane [61].

PGH_2 is the precursor of prostaglandins and thromboxanes. The synthesis of thromboxane A_2 (TXA_2), prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) from PGH_2 are catalyzed by TXA synthase, PGE synthase and PGI synthase, respectively [59]. These enzymes are found on the cytoplasmic face of the ER. PGH_2 formed in the lumen of ER via COXs diffuses across the membrane and is converted to a prostanoid end product on the cytoplasmic side of the membrane [59,61]. The major renal site for TXA_2 synthesis is glomerular podocytes and mesangial cells, PGE_2 is synthesized in juxtamedullary glomeruli, medullary interstitial cells and in the medullary portion of the collecting duct, and PGI_2 is most abundantly produced in the renal cortex by cortical arterioles and juxtamedullary glomeruli [52,53].

Renal prostanoids not only play an important role maintaining GFR, but also are involved in inflammatory processes in response to and as part of the renal injury [55,56]. Increased prostanoids are inflammatory mediators, as PGE_2 induces vasodilatation and increases vascular permeability; PGI_2 inhibits platelet aggregation and induces vasodilation; and TXA_2 induces vasoconstriction and promotes platelet aggregation [52,53,56]. Vasodilation caused by PGE_2 and PGI_2 increases renal blood flow, GFR and increases sodium excretion [53]; therefore, it plays essential roles maintaining renal vascular homeostasis and regulating salt and water homeostasis [53]. PGI_2 and PGE_2 also

stimulate renin secretion, activate the renin-angiotensin-aldosterone system which enhances sodium retention and potassium excretion [52,53].

1.3.2 Prostanoids and Autosomal Dominant Polycystic Kidney Disease

The kidney is a rich source of prostanoids that are known to regulate renal functions such as hemodynamics, water and solute transport, and renin secretion [52]. As previously described, renal prostanoids not only play an important role in maintaining renal vascular homeostasis and regulating salt and water homeostasis, but also are involved in inflammatory processes in response to and as part of the renal injury [55,56].

Vasodilators, PGE_2 and PGI_2 optimize renal blood flow, maintain GFR and preserve renal function [53,56]. Increased prostaglandin production has been demonstrated in patients with nephrotic syndrome [69] and CHF [54], and rats with experimental glomerulonephritis [70], reduced renal mass [71], and bilateral ureteral obstruction (BUO) [72]. Inhibition of prostaglandin synthesis resulted in reduced renal function in patients with nephritic syndrome and systemic lupus erythematosus (SLE) [69], and impairment of prostaglandin production is associated with acute reduction in renal blood flow and GFR in chronic renal insufficiency [53]. Intravenous administration of prostaglandins improved renal function in patients with chronic glomerulonephritis [69]. These studies support the importance and beneficial role of renal vasodilative prostaglandins in maintaining renal hemodynamics in renal diseases.

In addition, the vasoconstrictor TXA_2 which reduces GFR and worsens renal function is also increased in renal diseases. Studies show that TXA_2 production is increased in rats with reduced renal mass [71], experimental glomerulonephritis [70] and BUO [72], and

patients with nephritic syndrome [69] and CHF [54]. Increased TXA_2 production decreases renal blood flow and GFR [52], and selective TXA_2 synthase inhibition protects renal function and prevents histological damage in experimental glomerulonephritis [69]. Theses studies demonstrate the detrimental effects of renal TXA_2.

COX-1 and COX-2 are the key enzymes in the synthesis of prostaglandins and thromboxanes [59,60]. COX-1 is constitutively expressed in nearly all cell types at a constant level, serving homeostatic prostanoid synthesis [63,64,73], but can also be up-regulated by proinflammatory cytokines [67], while COX-2 is induced at sites of inflammation and produces the prostanoids involved in inflammatory responses, but low and detectable levels of COX-2 mRNA are also present in the normal kidney [63,74]. Glomerular mesangial cells play essential roles in maintaining renal hemodynamics and in immunologic functions of renal glomeruli [67,75]. One study was done to determine whether inflammatory stimulants can modulate COX expression and prostanoid production in glomerular mesangial cells [67]. Rat glomerular mesangial cells were incubated with several inflammatory stimulants such as interleukin-1β, growth-related oncogene-α and tumor necrosis factor-α for 0.5 to 24 h. Results showed that COX-1 mRNA and the protein levels of COX-1 were enhanced in the presence of inflammatory stimulants while detection of COX-2 was difficult. The production of PGE_2 and TXA_2 was increased in the presence of inflammatory stimulants. The authors concluded that inflammation stimulants enhance COX-1 but not COX-2 expression in cultured glomerular mesangial cells leading to increased renal PGE_2 and TXA_2 production [67].

In both the CD1-*pcy/pcy* mouse and the Han:SPRD-*cy* rat models of ADPKD, altered renal protein levels of prostanoid biosynthetic enzymes have been detected [76]. Immunoblotting analyses of cytosolic and particulate kidney fractions revealed that $cPLA_2$ levels were significantly higher in the latter stages of the disease in both models. Renal COX enzymes were found only in the particulate fractions with COX-1 significantly higher in 6-month-old CD1-*pcy/pcy* mice and 70-day-old male Han:SPRD-*cy* rats with diseased kidneys compared with controls. Renal COX-2 was detected only in the rats and was significantly lower in diseased kidneys of 70-day-old Han:SPRD-*cy* rats. With respect to prostanoid production, one study showed that PGE_2 production was decreased at 6 weeks of age in the Han:SPRD-*cy* rats model of ADPKD [51]. However, in this study, the PGE_2 production was based on net weight; since polycystic kidneys contain more water, it is difficult to determine whether the reduced renal PGE_2 production was caused by a reduction in renal tissue or by the disease itself. Therefore, the exact effect of disease on individual prostanoid production and COX isoform activities in the Han:SPRD-*cy* rats with renal disease is still not known.

1.3.3 Prostanoids and Dietary Interventions

Other than renal diseases, previous studies also demonstrated that renal prostanoid biosynthesis can be modulated by varying the composition of the diet. Ingesting a diet deficient in essential fatty acids resulted in reduced tissue levels of AA and the suppression of renal prostaglandin synthesis, whereas supplementation of the diet with fatty acids, such as linoleic acid and EPA, increases the synthesis of prostanoids [70]. In addition, previous studies showed that administration of indomethacin, a COX inhibitor,

blocks the increase in GFR that follows after a meat meal or the infusion of arginine [77,78]. This finding that the protein- or amino acid-induced rise in GFR can be blocked by a COX inhibitor suggests that the alterations of GFR may be mediated by prostanoids. Since dietary protein intervention can alter GFR, several studies were conducted to determine whether dietary protein intervention modulates changes in renal hemodynamics through effects on renal prostanoid production and the prostanoid biosynthetic enzymes.

One study was done to evaluate whether dietary protein intake could exert effects on glomerular prostanoid production in rats with remnant kidneys [79]. Rats were placed on either a high protein (51%) or a low protein (8.7%) diet for 2 weeks. Proteinuria, GFR and glomerular PGE_2, PGI_2 and TXA_2 production were significantly higher in rats on a high protein diet compared to a low protein diet. Moreover, TXA_2 formation was increased by only 40%, whereas PGE_2 and PGI_2 biosynthesis was increased by 2.5 and 12 times, respectively, in rats on a high protein compared to a low protein diet. This study not only demonstrated that high protein diet leads to increased prostanoid production, which is most evident on the vasodilatory prostaglandin production, but also suggested that the elevated prostaglandins might contribute to the increased GFR observed in rats on high protein diet.

Another study demonstrated that dietary protein intake modulates glomerular prostanoid production in both normal and diseased rats [80]. Normal and diseased rats received either a high protein diet (40%) or a low protein diet (8.5%) for 10-14 days. Glomeruli were isolated and incubated in the absence (basal) and presence (stimulated condition) of AA, and prostanoid production was measured. Under basal conditions,

glomerular production of PGE_2 and TXA_2 was significantly greater in rats ingesting the high protein diet. Glomerular production of PGE_2 and TXA_2 was also greater in animals fed the high-protein diet in the presence of AA, suggesting that glomerular COX activity was augmented. In addition, enalapril, an angiotensin-converting enzyme inhibitor, was used to investigate the potential role of the renin-angiotensin system in the dietary protein-induced modulation of glomerular prostanoid production in normal rats. Results showed that enalapril attenuated the dietary protein-induced augmentation in glomerular production of PGE_2 and TXA_2. The authors concluded that dietary protein modulates glomerular PGE_2 and TXA_2 synthesis in the rats and this effect appears to require a functioning renin-angiotensin system.

The alterations of glomerular prostanoid production caused by dietary protein intake and the importance of renin-angiotensin system in these changes were examined in BUO rats as well [72]. BUO and sham-operated control (SOC) rats were fed either a low protein (4%) or a high protein (40%) diet for approximately 4 weeks. Rats were pre-treated or not with the angiotensin converting enzyme inhibitor, enalaprilat, prior to sham-operation or ureteral obstruction. Glomeruli from SOC rats fed a high protein diet produced significantly greater amounts of PGE_2, PGI_2 and TXA_2, and had substantially increased protein levels of COX when compared with glomeruli from SOC rats fed a low protein diet. Rats pre-treated with enalaprilat prior to sham operation prevented the increase in glomerular prostanoid production and COX levels in SOC rats fed a high protein diet. Both prostanoid production and COX levels were further increased in glomeruli from rats with BUO fed a high protein diet when compared with glomeruli of SOC rats fed the same diet. The increased levels of these measurements in BUO rats fed

a high protein diet fell markedly when the rats were pre-treated with enalaprilat. In addition, there was no substantial increase in prostanoid production and the levels of COX in glomeruli of BUO versus SOC rats fed a low protein diet. Enalaprilat did not affect glomerular prostanoid production or COX levels in SOC and BUO rats fed a low protein diet. This study indicated that dietary protein affects glomerular prostanoid production in BUO rats by altering the activity of the COX pathway mainly via the renin-angiotensin system.

Results from these studies indicate that high protein intake may alter renal hemodynamics through increased COX activity and increased production of prostanoids as a consequence of activated renin-angiotensin system. More importantly, previous studies also showed that not all types of protein share the same capacity to raise GFR [58,81]. In one study, healthy individuals were fed either an animal protein or a soy protein diet for 3 weeks, and these two diets contained the same amount of total protein [58]. Results showed that protein intake did not differ between groups; GFR and urinary PGI_2 were significantly higher in individuals on the animal protein diet than the soy protein diet. Authors suggested that soy protein has significantly different renal effects from animal protein in normal humans which may be explained by differences in renal vasodilatory prostaglandin secretion.

As to the renal effects of soy protein in renal disease, a recent study showed that in the Han:SPRD-*cy* rat model of ADPKD, 1-3 weeks of soy protein feeding compared with casein ameliorates the suppression of PGE_2 production in the early stage of renal disease [51], and another study showed that 8 weeks of CLA feeding reduces renal PGE_2 production in the same animal model [26]. However, the exact effect of dietary soy

protein on renal individual prostanoid production and the protein levels and activities of prostanoid biosynthetic enzymes have not been examined in this model of PKD.

1.4 Preliminary Study

1.4.1 Rationale

Dietary soy protein intervention delays disease progression in weanling Han:SPRD-*cy* rats after consuming the diet for as little as 1-3 to 8 wk [48,49,50,51,82,83]. Previous studies also demonstrate that disease progression in the latter stages of ADPKD is less influenced by diet [84], and greater beneficial effects are observed when dietary interventions were introduced at weaning [23,39,48,49]. Components of soy protein, such as genistein, can cross placenta and be passed on to the fetus directly and to the suckling animals via altered milk composition in Sprague Dawley rats [85,86]. Since ADPKD begins as early as in *utero* and progresses slowly through the growing stages and into adulthood, and maternal diet influences the fetus as well as milk composition [85,86], it is reasonable to assume that dietary soy protein intervention in pre-weaning stages may also be effective, and perhaps even more so. However, the effect of initiating dietary soy protein intervention in *utero* and during lactation by manipulating the maternal diet has not been studied before.

In addition, the kidney is a rich source of prostanoids which regulate renal hemodynamics, water and solute transport, and renin secretion [52]. Prostanoids not only play a role in maintaining GFR, but also are involved in inflammatory processes in response to and as part of the renal injury [55,56]. Renal prostanoid production has been shown to be altered by diseases and dietary interventions [53,54,72,79,80]. In the

Han:SPRD-*cy* rat model of ADPKD, protein levels of $cPLA_2$ and both COX-1 and COX-2 isoforms are altered in diseased kidneys compared to normals [76].

Moreover, studies showed that in the Han:SPRD-*cy* rat model of ADPKD, including CLA for 8 weeks in the diets not only retards early disease progression, but also reduces inflammation and the production of one prostanoid, PGE_2 [26]. Including soy protein in the diets for only 1-3 weeks reduces renal cyst and fibrosis, and ameliorates the suppression of PGE_2 production in the early stage of renal disease [51]. Including soy protein in the diets for 6-8 weeks improves renal function, reduces cyst growth, renal fibrosis and inflammation [48,83]. It is known that soy protein compared to meat protein has a reduced effect on renal hyperfiltration and proteinuria. These effects appear to be mediated by lowered renal prostanoid production, among other effects [58]. However, if the delayed disease progression and improved renal function caused by dietary soy protein intervention in the Han:SPRD-*cy* rat model of ADPKD are associated with alterations in prostanoid production and prostanoid biosynthetic enzymes are not known.

Therefore, in our preliminary study, the objectives were to examine the effects of dietary soy protein in the maternal diet (initiated before conception, during pregnancy and lactation) on progression of renal injury in the Han:SPRD-*cy* rat model of ADPKD, and to examine the effects of disease and dietary soy protein intervention in both the maternal and post-weaning diet on the protein levels of renal prostanoid biosynthetic enzymes, namely, $cPLA_2$, COX-1 and COX-2.

1.4.2 Hypotheses of Preliminary Study

1. Dietary soy protein intervention will delay progression of renal injury in the offspring of Han:SPRD-*cy* rat model of ADPKD if it is initiated in the maternal diet.
2. The beneficial effects of dietary soy protein intervention in both the maternal and post-weaning diet are associated with the alterations in the protein levels of renal prostanoid biosynthetic enzymes, namely, $cPLA_2$, COX-1 and COX-2.

1.4.3 Animal Model and Dietary Interventions

Han:SPRD-*cy* rats were obtained from our breeding colony that is derived from animals that were provided by Dr. B.D. Cowley (University of Kansas Medical Center, Kansas City, KS, USA). The experimental protocol was in accordance with Canadian Council of Animal Care guidelines and was approved by the University of Manitoba Animal Care and Use Committee. The heterozygous Han:SPRD-*cy* (*cy*/+) rat which resembles human ADPKD, is a well-documented animal model of ADPKD [5,7]. Food and water were provided *ad libitum*. Diets were based on the AIN 93G laboratory rodent diet [87] modified with corn oil as the lipid source (Table 1). The soy protein diet (20% soy protein) was exactly the same as the casein diet (20% casein), except that casein was replaced with an equivalent amount of protein from heat-treated soy protein isolate (Dyets, Inc., Bethlehem, Pennslyvania) in order to maintain equal energy density of the diets.

Table 1. Composition of Casein and Soy Protein Diets

Ingredient	Casein Diet (g/kg of diet)	Soy Protein Diet (g/kg of diet)
Cornstarch	397.5	397.5
Casein (87% protein) [b]	200.0	0
Soy Protein (87% protein) [b]	0	200.0
Maltodextrin [c]	132.0	132.0
Sucrose [c]	100.0	100.0
Cellulose (Fiber) [c]	50.0	50.0
AIN-93G Mineral Mix [c]	35.0	35.0
AIN-93VX Vitamin Mix [c]	10.0	10.0
L-Cystine [b]	3.0	3.0
Corn Oil [a,b]	70.0	70.0
Choline Bitartrate [c]	2.5	2.5
Total	1000.0	1000.0

Diets were based on the AIN-93G [87] purified rodent diet modified with corn oil as the lipid source.

[a] Contains 0.02% tert-butylhydroquinone (TBHQ).

[b] Supplied by Dyets, Inc., Bethlehem, Pennslyvania.

[c] Supplied by Harlan Teklad, Madison, Wisconsin.

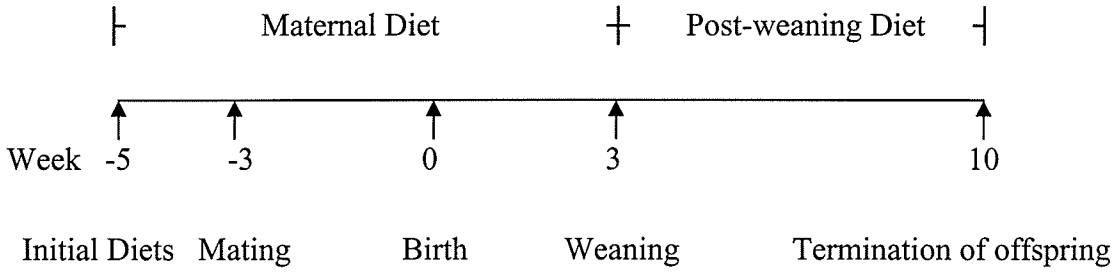
Dams and sires were maintained on the experimental diets for at least two weeks prior to mating, during pregnancy (3 weeks) and lactation (3 weeks). The nutritional environment during this time period is referred to as the maternal diet in this thesis. Weaned pups were then given experimental diets for 7 weeks (referred to as the post-weaning diet), for a total experimental period of 15 weeks. Dams and their litters were divided into 4 groups and given diets as follows in a 2 × 2 design:

(1) Casein diet for entire 15 weeks (AC/AC)

(2) Casein diet from week -5 to 3 (weaning) and soy protein diet from week 3 to 10 (AC/SC)

(3) Soy protein diet from week -5 to 3 (weaning) and casein diet from week 3 to 10 (SC/AC)

(4) Soy protein diet for entire 15 weeks (SC/SC)



Only male weanlings were used in this study because the progression of renal injury is more rapid in males compared to females and differences are more likely to be detected. Both normal and heterozygous rats were maintained on the diet for seven weeks after weaning, a common endpoint for studies in these animals, and the usual length of feeding in our previous feeding trials [50]. Normal and heterozygous rats were

differentiated from each other by histological analyses after termination. Body weights were monitored weekly. Three and six weeks after weaning, animals were put into metabolic cages for 2 days before 24-hour food and water intake, and urine volume were measured. At the end of the feeding periods, animals were quickly anesthetized with carbon dioxide and decapitated. Trunk blood was collected and centrifuged at 4500 × *g* for 15 minutes to obtain serum. Renal functional changes were determined by measuring serum and urine creatinine, creatinine clearance, serum urea nitrogen (SUN), and urine protien.

Kidneys were removed and weighed. Left kidneys were fixed in a 10% formalin solution. Histological and morphometric changes, including cyst volume, fibrous volume, proliferating cell nuclear antigen (PCNA) count and macrophage count (inflammation) were measured. Right kidneys were immediately frozen in liquid nitrogen and stored at –80°C for analyses of the steady-state levels of $cPLA_2$, COX-1 and COX-2 by using western immunoblotting [76]. For method details of western immunoblotting, please see the appendix 5.1.

1.4.4 Statistical Analyses

From previous experience with the Han:SPRD-*cy* rats [48,51,76,82,83] and the expected variance in histological changes and in protein expression, we expected a power of at least 80 to be achieved with 10-15 rats in each group.

Normality of the data was tested using the Shapiro-Wilk statistic ($W > 0.01$ for normality) and log transformed if it did not follow a normal distribution. For normally distributed data, results for weight, food and water intake, renal function analyses and

histological and morphometric analyses were analyzed by 2×2 ANOVA (maternal diet × weaning diet), results for western immunoblotting were analyzed by 2×2×2 ANOVA (genotype × maternal diet × weaning diet) using SAS software (SAS Institute, Cary, North Carolina). Main effects and simple differences were considered significant at $P < 0.05$ and interactions at $P < 0.10$. If normality was not achieved after log transformation, differences were determined using non-parametric analysis (Wilcoxin rank sum test, $P < 0.10$). All data are expressed as means ± the standard error of the mean (SEM).

1.4.5 Results

1.4.5.1 Body Weight, Food and Water Intake

No difference was found with respect to final body weight, food and water intake between diseased rats that consumed control or soy protein in the maternal and/or post-weaning diets. (Table 2)

1.4.5.2 Renal Function Analyses

As to the renal functional measurements (Table 3), data showed that in diseased rats, soy protein in the post-weaning diet resulted in 23% higher urine creatinine ($p = 0.0054$), 29% lower serum creatinine ($p < 0.0001$), 72% higher creatinine clearance ($p < 0.0001$) and 45% lower serum urea nitrogen (SUN) ($p < 0.0001$) compared with control diet. Dietary soy protein in the maternal diet resulted in 35% lower urine protein relative to urine creatinine ($p = 0.0013$).

1.4.5.3 Histological and Morphometric Analyses

Histological and morphometric analyses of left kidneys showed that in diseased rats, soy protein in the post-weaning diet resulted in 12% lower left kidney weight relative to body weight ($p = 0.0020$), 21% lower cyst scores ($p = 0.0014$) and 30% lower macrophage count ($p < 0.0001$). Fibrosis volume was lower in diseased rats fed soy protein in the post-weaning diet. However, the difference was not statistically significant ($p = 0.0635$).

Dietary soy protein in the maternal diet independently resulted in 22% lower macrophage count ($p = 0.0003$). In addition, there was a maternal diet by post-weaning diet interaction found for proliferating cell nuclear antigen (PCNA) count ($P = 0.0040$). Rats fed soy protein in the maternal and/or post-weaning diet had lower PCNA score than rats fed control diet in both the maternal and post-weaning diets (Table 4).

Table 2. Effects of soy protein in the maternal and post-weaning diets on final body weight and food and water intake in the Han:SPRD-*cy* rats with ADPKD

Maternal Diet	AC		SC		Effects
Post-weaning Diet	AC	SC	AC	SC	
Final Body Weight (g)	333.3 ± 5.4[1]	329.1 ± 3.2	321.5 ± 7.0	324.3 ± 4.1	None
Food Intake (g/day) (3 wk)	15.9 ± 0.4	16.6 ± 0.6	15.2 ± 0.9	15.5 ± 0.8	None
Food Intake (g/day) (6 wk)	15.2 ± 0.8	15.3 ± 0.9	14.1 ± 1.0	15.0 ± 0.8	None
Water Intake (g/day) (3 wk)	22.9 ± 0.8	25.0 ± 0.9	26.8 ± 1.4	24.9 ± 0.8	None
Water Intake (g/day) (6 wk)	35.7 ± 2.6	30.8 ± 1.5	33.1 ± 1.7	31.8 ± 1.5	None

[1] Data is presented as mean ± SEM. (n = 10-15)

Table 3. Effects of soy protein in the maternal and post-weaning diets on kidney function in the Han:SPRD-*cy* rats with ADPKD

Maternal Diet	AC		SC		Effects
Post-weaning Diet	AC	SC	AC	SC	
Urine Creatinine[2] (mmol/L)	4.08 ± 0.40[1]	5.48 ± 0.30	4.95 ± 0.00	5.68 ± 0.34	**Post-weaning Diet** (P = 0.0054)
Serum Creatinine[3] (µmol/L)	72.16 ± 3.52	51.92 ± 2.64	79.2 ± 5.28	54.56 ± 2.64	**Post-weaning Diet** (P < 0.0001)
Serum Urea[4] (mmol/L)	33.93 ± 2.46	18.25 ± 1.58	31.53 ± 2.64	17.85 ± 1.20	**Post-weaning Diet** (P < 0.0001)
Creatinine Clearance (mL/min)	0.671 ± 0.061	1.278 ± 0.084	0.823 ± 0.089	1.293 ± 0.086	**Post-weaning Diet** (P < 0.0001)
Urine Protein/Urine Creatinine (mg/mg)	1.41 ± 0.12	1.65 ± 0.22	0.97 ± 0.10	1.08 ± 0.10	**Maternal Diet** (P = 0.0013)

[1] Data is presented as mean ± SEM. (n = 10-15)
[2] To convert to mg/dL, divide by 0.088.
[3] To convert to mg/dL, divide by 88.
[4] To convert to serum urea nitrogen (mg/dL), multiply by 2.8.

Table 4. Effects of soy protein in the maternal and post-weaning diets on histological changes in the Han:SPRD-*cy* rats with ADPKD

Maternal Diet	AC		SC		Effects
Post-weaning Diet	AC	SC	AC	SC	
Kidney Weight /Body Weight (g/100g)	13.1 ± 0.4[1]	11.8 ± 0.6	13.7 ± 0.7	11.7 ± 0.4	**Post-weaning Diet** (P = 0.0020)
Cyst Score[a]	5.59 ± 0.32	4.72 ± 0.45	6.43 ± 0.47	4.75 ± 0.28	**Post-weaning Diet** (P = 0.0014)
Fibrosis Area (ml/kg body wt)	0.027 ± 0.003	0.021 ± 0.003	0.032 ± 0.004	0.026 ± 0.002	**No Effect** (P = 0.0635)
PCNA[b] Count (cells/ mm epithelium)	38.4[a,2] ± 1.5	26.3[b] ± 1.7	27.2[b] ± 1.4	24.0[b] ± 1.1	**Maternal × Post-weaning Diet**
Macrophage Count (cells/ high power field)	34.2 ± 2.3	23.4 ± 1.4	26.1 ± 2.2	17.8 ± 0.8	**Maternal Diet** (P = 0.0003) **Post-weaning Diet** (P < 0.0001)

[1] Data is presented as mean ± SEM. (n = 11-15)
[2] Numbers in rows that contain different superscripts are significantly different.
[a] Cyst area (%) × left kidney weight (g)/body weight (g).
[b] PCNA, proliferating cell nuclear antigen

1.4.5.4 Western Immunoblotting

The results from western immunoblotting showed that in diseased compared to normal rats, renal protein levels of $cPLA_2$ were 34% ($P = 0.0139$) and 59% higher ($P = 0.0105$) in the cytosolic and particulate fractions, respectively. In the particulate fractions, COX-1 was 28% higher ($P = 0.0311$) and COX-2 was 95% lower ($P < 0.0001$) in diseased kidneys compared to normals.

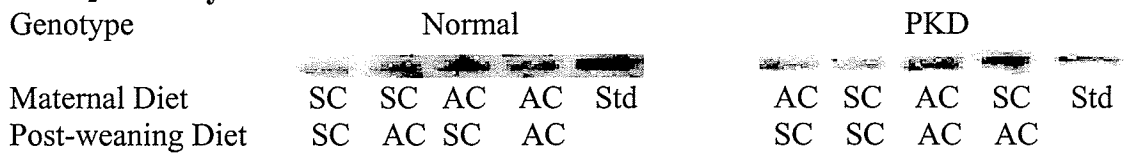
The elevated $cPLA_2$ in the particulate fraction of diseased kidneys was reduced by 46% ($P = 0.0087$) in the rats given soy protein compared to casein in the post-weaning diet. Soy protein in the maternal diet did not alter the protein levels of the other prostanoid biosynthetic enzymes (Table 5, Figure 1).

Table 5. Effects of soy protein in the maternal and post-weaning diets on steady-state protein levels of $cPLA_2$, COX-1 and COX-2 in the Han:SPRD-*cy* rats

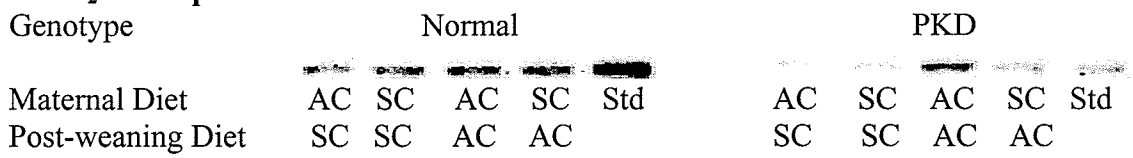
Maternal Diet		AC		SC		Effects
Post-weaning Diet		AC	SC	AC	SC	
Cytosolic $cPLA_2$ (arbitrary units)	Normal	60.18 ± 8.25[1]	67.95 ± 8.82	81.79 ± 13.91	69.23 ± 8.18	**Genotype** ($P = 0.0139$)
	PKD	80.81 ± 10.95	92.15 ± 18.76	99.05 ± 13.18	101.14 ± 16.84	
Particulate $cPLA_2$ (arbitrary units)	Normal	43.85[a,2] ± 6.07	64.75[a] ± 15.84	55.91[a] ± 9.72	42.55[a] ± 8.11	**Geno × Post-weaning Diet**
	PKD	102.25[b] ± 14.96	55.25[a] ± 25.07	109.43[b] ± 14.25	62.99[a] ± 11.39	
Particulate COX-1 (arbitrary units)	Normal	73.85 ± 4.06	85.61 ± 13.53	85.13 ± 6.40	83.47 ± 11.93	**Genotype** ($P = 0.0311$)
	PKD	102.64 ± 12.67	90.13 ± 7.13	125.58 ± 15.60	102.13 ± 19.70	
Particulate COX-2 (arbitrary units)	Normal	186.77 ± 18.30	225.53 ± 23.80	233.83 ± 20.05	209.48 ± 27.96	**Genotype** ($P < 0.0001$)
	PKD	11.82 ± 4.12	11.12 ± 2.91	8.37 ± 2.88	7.84 ± 2.48	

[1] Data is presented as mean ± SEM. (n = 8-9)
[2] Values within maternal diet groups which contain different superscripts are significantly different.

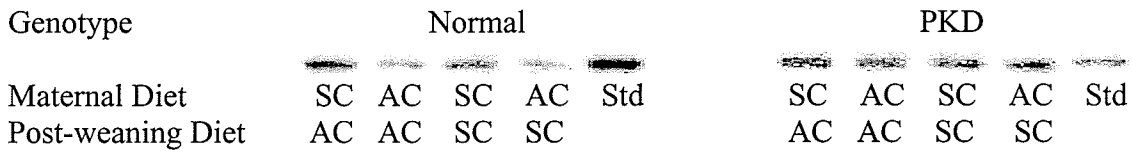
$cPLA_2$ in the cytosolic fractions



$cPLA_2$ in the particulate fractions



COX-1



COX-2

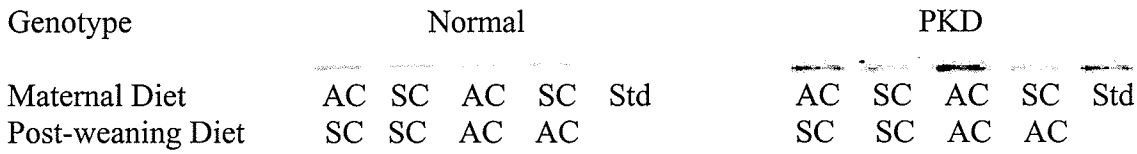


Figure 1. Representative immunoblots of prostanoid biosynthetic enzymes in normal and diseased in Han:SPRD-*cy* rats. Std, standard. (The same sample from a diseased rat was used as Std on all gels)

1.4.6 Discussion of Preliminary Results

Data from our preliminary study showed that, consistent with previous studies [48,83], dietary soy protein in the post-weaning diet significantly ameliorated disease progression, improved renal function and reduced renal inflammation in the Han:SPRD-*cy* rat model of ADPKD. Dietary soy protein in the maternal diet also independently reduced renal inflammation and proteinuria. Soy protein in either the maternal or post-weaning diet reduced renal cell proliferation, with either intervention being equally effective. Therefore, in addition to confirming that dietary soy protein in the post-weaning diet retards renal disease progression, this study demonstrates that dietary soy protein in the maternal diet provides further benefits in offspring with renal disease.

With respect to the effect of renal disease on the protein levels of prostanoid biosynthetic enzymes, data from this preliminary study are consistent with a previous study [76]. Renal protein levels of $cPLA_2$ in the cytosolic and particulate fractions and COX-1 were significantly elevated while COX-2 was significantly decreased in diseased compared to normal rats. Dietary soy protein in the post-weaning diet compared to casein resulted in lower protein levels of $cPLA_2$ in the particulate fraction of diseased kidneys but did not change the protein levels of $cPLA_2$ in the cytosolic fraction, COX-1 or COX-2. Dietary soy protein in the maternal diet did not alter the protein levels of any prostanoid biosynthetic enzymes. The lower levels of $cPLA_2$ in the particulate fraction of diseased kidneys given dietary soy protein in the post-weaning diet indicates that less activated $cPLA_2$ is available for releasing AA from cell membranes, resulting in less substrate for COX isoforms to produce prostanoids.

Previous studies demonstrate that renal production of PGE_2, PGI_2 and TXA_2 is increased by disease [54,69,70,71,72]. The vasodilators, PGE_2 and PGI_2 optimize renal blood flow, increase GFR and play important roles in maintaining renal hemodynamics in diseased kidneys while the vasoconstrictor, TXA_2 reduces GFR and worsens renal function [52,53,56]. Both COX-1 and COX-2 can be induced in inflammation [63,67]; however, one previous study showed that incubating rat glomerular mesangial cells with inflammation stimulants enhances COX-1 but not COX-2 expression leading to increased renal PGE_2 and TXA_2 production [67]. Mesangial cells synthesize dominantly the constitutive isoform of COX-1 rather than COX-2 to fulfill the physiological requirement of the kidney. In the current study, the protein levels of COX-1 are elevated while the protein levels of COX-2 are significantly decreased in the diseased Han:SPRD-*cy* rats [76]. It is reasonable to assume that in the diseased Han:SPRD-*cy* rats, COX-1 rather than COX-2 is involved in the increased renal prostanoid production and that not only the protein levels of COX-1, but the activity of COX-1 may also be elevated. However, the effect of renal disease on renal prostanoid production and COX isoform activities in the Han:SPRD-*cy* rats is not known.

Data from our preliminary study show that dietary soy protein in the post-weaning diet compared to the maternal diet has greater effects on preserving renal function and delaying disease progression. In addition, soy protein in the post-weaning diet reduces the elevated protein levels of $cPLA_2$ in the particulate fractions in the diseased kidneys while soy protein in the maternal diet did not alter the protein levels of any of the prostanoid biosynthetic enzymes. Since PGE_2 and PGI_2 are essential for maintaining renal hemodynamics and TXA_2 has detrimental effects in diseased kidneys [56], the beneficial

effects of dietary soy protein in the post-weaning diet may be due to selective reduction of the detrimental TXA_2 without altering the beneficial PGE_2 and PGI_2. In addition, in diseased Han:SPRD-*cy* rats, COX-1 rather than COX-2 appears to be involved in the increased renal prostanoid production. Dietary soy protein in the post-weaning diet does not change the protein levels of COX-1 or COX-2; therefore, the beneficial effects of dietary soy protein in the post-weaning diet may be due to the reduction of COX-1 activity. However, whether the delayed disease progression and improved renal function caused by dietary soy protein in the post-weaning diet in diseased Han:SPRD-*cy* rats are associated with alterations in prostanoid production and COX isoform activities is not known.

1.5 Objective and Hypotheses

1.5.1 Objective

Therefore, in our study, the objective was to determine whether disease and dietary soy protein in the post-weaning diet have effects on renal prostanoid production and the COX isoform activities in the Han:SPRD-*cy* rat model of ADPKD. The rats in the post-weaning portion of the preliminary study were chosen for these analyses because the dietary soy protein effects on disease progression were greatest during this time period.

1.5.2 Hypotheses

We hypothesized that in the Han:SPRD-*cy* rat model of ADPKD,

1. Disease increases renal PGE_2, PGI_2 and TXA_2 production and selectively increases COX-1 activity.

2. Soy protein in the post-weaning diet selectively decreases renal TXA_2 production and COX-1 activity.

These hypotheses were tested and results are presented in the next chapter in manuscript format.

2. Dietary soy protein and disease selectively alter cyclooxygenases and prostanoid production in the Han:SPRD-*cy* rat model of polycystic kidney disease

ABSTRACT

Background Soy protein slows disease progression in the weanling Han:SPRD-*cy* rat model of polycystic kidney disease. Alterations in prostanoid biosynthetic enzymes are present in the kidneys of these rats. The objective of this study was to examine the effects of disease and dietary soy protein on renal prostanoid production and COX isoform levels and activities.

Methods Weanling Han:SPRD-*cy* rats were given casein or soy protein diets for 7 weeks. Renal protein levels of cytosolic and particulate $cPLA_2$, COX-1 and COX-2 were determined by immunoblotting. Renal prostanoid production and COX isoform activities were determined by EIA, using a selective COX inhibitor.

Results Renal prostanoid production and activities of both COX isoforms were significantly elevated in diseased kidneys compared to normals. COX-2 was the predominant COX activity in both normal and diseased kidneys. The effect of disease on the levels of prostanoids, as well as the COX activities producing specific prostanoids was in the order of TXA_2> PGI_2> PGE_2. In diseased kidneys, dietary soy protein feeding lowered the *in vitro* steady-state production of TXA_2 by 49% ($P = 0.0054$), and resulted in less TXA_2 and PGI_2 produced by COX-2 activity by 47% ($P = 0.0030$) and 36% ($P = 0.0195$), respectively, and less PGI_2 produced by COX-1 activity by 76% ($P = 0.0011$). The effect of soy protein feeding on COX activity was in the order of TXA_2> PGI_2> PGE_2.

Conclusion Renal prostanoids and prostanoid biosynthetic enzymes are selectively altered by renal disease in the Han:SPRD-*cy* rat, and the beneficial effects of dietary soy protein in this form of renal injury are associated with specific attenuations of the disease effects on prostanoids.

Key Words: Soy protein; Kidney disease; Prostanoids; Cyclooxygenases; TXA_2; PGI_2; PGE_2

INTRODUCTION

The kidney produces a number of prostanoids that are known to regulate renal functions such as hemodynamics, water and solute transport, and renin secretion [52]. Renal prostanoid production is initiated by arachidonic acid (AA) release from cellular lipid membranes, followed by conversion to PGH_2 by COX-1 or COX-2, with subsequent conversion to the prostanoids thromboxane A_2 (TXA_2), prostaglandin I_2 (PGI_2), and prostaglandin E_2 (PGE_2). In healthy kidneys, prostanoids maintain normal renal homeostasis, while in diseased kidneys, they play a role in maintaining glomerular filtration rate (GFR), as well as being involved in inflammatory processes in response to and as part of the renal injury [55,56].

Autosomal dominant polycystic kidney disease (ADPKD) is a renal disorder that is characterized by abnormal growth and development of renal cysts, interstitial fibrosis and renal inflammation. It is the most commonly inherited kidney disease, affecting approximately 600,000 US citizens and an estimated 12.5 million people worldwide [1]. ADPKD accounts for 8-10% of the cases of ESRD and costs for dialysis and transplantation exceed more than $1.5 billion annually in the United States [1]. Mutations in *PKD1* or *PKD2* genes are present in most ADPKD patients; an unidentified mutated gene *PKD3* has been implicated in a small number of patients [4,9,11].

Treatment of ADPKD with pharmacological or dietary treatments which impede inflammation may offer a means of delaying the progression of this disease. Methylprednisolone is an anti-inflammatory drug with antifibrogenic effects which slows progression of ADPKD in rodent models [17]. A number of dietary interventions with potential anti-inflammatory effects also retard early disease progression in animal models

of ADPKD [21,26,48,49,50,83]. Including CLA for 8 weeks in the diets of Han:SPRD-*cy* rats not only retards early disease progression, but also reduces inflammation and the production of one prostanoid, PGE_2 [26]. Including soy protein in the diets of rodent models of ADPKD for 6-8 weeks reduces cyst growth, renal fibrosis and inflammation [48,49,50]. It is known that soy protein compared to meat protein has a reduced effect on renal hyperfiltration and proteinuria. These effects appear to be mediated by lowered renal prostanoid production, among other effects [57,58]. However, the effects of dietary soy protein on prostanoid production in ADPKD are not known.

Renal prostanoid production is increased in a number of diseases of the kidney, such as chronic renal insufficiency and nephritic syndrome [53]. In the Han:SPRD-*cy* rat model of ADPKD, protein levels of $cPLA_2$ and both COX-1 and COX-2 isoforms are altered in diseased kidneys compared to normals [76]. However, the contributions of each COX isoform to total COX activity or whether specific prostanoids are altered in this renal disease is not known. Therefore, the objective of the current study was to determine the effect of disease and dietary soy protein on COX isoform activity and prostanoid production in kidneys affected by ADPKD.

MATERIALS AND METHODS

Animals and Diets

Han:SPRD-*cy* rats were obtained from our breeding colony that is derived from animals that were provided by Dr. B.D. Cowley (University of Kansas Medical Center, Kansas City, KS, USA). The experimental protocol was in accordance with Canadian Council of Animal Care guidelines and was approved by the University of Manitoba Animal Care and Use Committee.

Male rats that were from casein-fed dams were weaned at 3 weeks of age, randomly divided into 2 groups, and given a 20 g/100 g soy protein diet or a 20 g/100 g casein diet for 7 weeks. Diets were based on the AIN 93G laboratory rodent diet [87] modified with corn oil as the lipid source (Table 1). The two diets were identical in composition with the exception of the protein sources as previously described [82]. At the end of the feeding period, animals were quickly anesthetized with carbon dioxide and decapitated. The kidneys were removed, weighed, and immediately frozen in liquid nitrogen and stored at -80°C until analyses.

Laboratory Analyses

Western immunoblotting

Steady-state levels of $cPLA_2$, COX-1 and COX-2 protein expression were determined as described [76]. Briefly, twenty mg of lyophilized kidneys were homogenized in 100 volumes of ice-cold homogenization buffer containing protease inhibitors. Homogenates were centrifuged at 100,000 *g* for 30 min at 4°C and the supernatant was removed and collected as the cytosolic fraction. The remaining pellet was resuspended in 20 volumes of homogenization buffer containing 10% Triton X-100 (Sigma, St. Louis, Missouri), incubated on ice for 10 min and centrifuged again at 100,000 *g* for 30 min at 4°C. The resulting supernatant was collected as the particulate fraction, and all samples were stored at -80°C. Protein concentrations were determined by protein assay as described by Bradford [88], with bovine serum albumin (Sigma, St. Louis, Missouri) as the standard.

Following SDS-PAGE, proteins were transferred to PVDF membranes, blocked and incubated with antibodies to $cPLA_2$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA), COX-1 and COX-2 (Cayman Chemical Company, Ann Arbor, MI). Immunoblots were

incubated with ChemiGlow™ (Alpha Innotech, San Leandro, CA). Image analysis and quantitation of immunoreactive bands were performed using the Flurochem Imager (Alpha Innotech, San Leandro, CA), as detailed [76]. For details of western immunoblotting methods, please see the appendix 5.1.

Renal COX activity

Sixty mg of lyophilized right kidney from each rat was homogenized on ice using 1.7 ml fresh Tyrodes buffer (Sigma, St. Louis, Missouri). After homogenization, 17 μL of 1% Triton was added, vortexed for 10 sec and the homogenate was placed on ice for 30 min. Every 10 min during this 30 min time period the homogenate was vortexed for 10 sec. Then, 180 μL of homogenate was aliquoted in duplicate into chilled micorcentrifuge tubes containing 20 μL Tyrodes buffer with vehicle (1% ethanol) or 0.1 μM SC560, a COX-1 selective inhibitor (Cayman Chemical Company, Ann Arbor, MI), and incubated for another 30 min on ice. The following four conditions were then used: 1) time 0 with no inhibitor, to determine endogenous kidney levels of eicosanoids; 2) 60 min incubation at 37°C with no inhibitor, to determine *in vitro* steady-state levels of eicosanoids; 3) 10 min at 37°C with no inhibitor, to determine total COX activity; and 4) 10 min incubation at 37°C with 0.1 μM SC560, to determine COX-2 activity. COX-1 activity was calculated by the difference between total COX (condition 3) and COX-2 (condition 4) activities. The incubation times and concentrations of SC560 were determined from previous time course studies (Warford et al, manuscript in preparation) which showed that steady-state levels of eicosanoids were achieved by 30-40 min of incubation and that 0.1 μM SC560 inhibits > 90% of COX-1 activity under these conditions, while not inhibiting COX-2 at all. After the specified incubation period, 800 μL of fresh, ice cold

5mmol/L acetylsalicylic acid (ASA) (Sigma, St. Louis, Missouri) was added to inhibit further COX activity. Samples were vortexed for 5 s and centrifuged at 12,000 *g* at 4°C for 5 min. The supernatant was removed and stored at -80°C until analysis using commercial enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI) for PGE_2 and the stable metabolites of TXA_2 (TXB_2) and PGI_2 (6-keto $PGF_{1\alpha}$). Samples were diluted between 100 times and 1500 times. Twenty μL of homogenate was set aside for protein determination using the Bradford assay [88].

Statistical analyses

Normality of the data was tested using the Shapiro-Wilk statistic ($W > 0.01$ for normality) and log transformed if it did not follow a normal distribution. For normally distributed data, results were analyzed by 2×2 ANOVA (genotype×diet) using SAS software (SAS Institute, Cary, North Carolina). Main effects and simple differences were considered significant at $P < 0.05$ and interactions at $P < 0.10$. If normality was not achieved after log transformation, differences were determined using non-parametric analysis (Wilcoxin rank sum test, $P < 0.10$). All data are expressed as means ± the standard error of the mean (SEM).

RESULTS

Dietary soy protein compared to casein retarded renal disease progression, reduced renal fibrosis, cyst growth, inflammation, cell proliferation and oxidation (Tables 3,4), consistent with what we have previously reported [48,83]. Also consistent with our previous results on enzyme levels in the Han:SPRD-*cy* rat, $cPLA_2$ and COX-1 levels were higher and COX-2 levels were lower in diseased kidneys. However, in the current analyses, the differences in COX-1 and $cPLA_2$ in the cytosolic fraction were not

statistically significant (Table 6). Dietary soy protein significantly attenuated the higher steady-state levels of $cPLA_2$ in the particulate fraction in the diseased kidneys, while $cPLA_2$ in the cytosolic fraction and the COX enzyme levels were not altered by diet.

Prostanoid production was higher in the diseased kidneys compared to normals. Endogenous and *in vitro* steady-state levels of prostanoids were ~5 times and ~10 times higher for TXA_2 (Table 7), ~2 times higher for PGE_2 (Table 8), ~4 times and ~5 times higher for PGI_2 (Table 9) and ~3 times and ~5 times higher for total prostanoids (Table 10) in diseased compared to normal kidneys. The effect of dietary soy protein on these parameters was specific for *in vitro* steady-state levels of TXA_2, which was 49% lower in diseased kidneys from rats fed soy protein compared to casein (Table 7).

COX activity in normal kidneys was predominantly (90-100%) due to the COX-2 isoform (Table 7-10). Total COX activity was higher in diseased kidneys when determined by production of any individual prostanoid (Tables 7-9) or the total of these prostanoids (Table 10). Compared to normal rat kidneys, total COX activity was 2-9 times higher, depending on the prostanoid used to determine activity: for TXA_2, PGE_2 and PGI_2, the elevations were 9 times, 2 times and 6 times, respectively. The activity of both COX isoforms also was elevated in diseased kidneys when determined by individual or total prostanoid production, except for COX-1 activity when determined by PGE_2 production (Table 8). For COX-1, activity as determined by TXA_2, or PGI_2 or total prostanoid production was 11-14 times higher and for COX-2, activity ranged from 2-8 times higher when determined by the individual or total prostanoids (Tables 7-10).

Table 6. Effects of casein compared to soy protein based diet on steady-state levels of $cPLA_2$, COX-1 and COX-2 in the Han:SPRD-*cy* rats

Genotype	Normal		PKD		Effects
Diet	**Casein**	**Soy Protein**	**Casein**	**Soy Protein**	
Cytosolic $cPLA_2$ (arbitrary units)	60.18 ± 8.25[1]	67.95 ± 8.82	80.81 ± 10.95	92.15 ± 18.76	**None**
Particulate $cPLA_2$ (arbitrary units)	43.85[a,2] ± 6.07	64.75[a] ± 15.84	102.25[b] ± 14.96	55.25[a] ± 25.07	**Geno × Diet**
Particulate COX-1 (arbitrary units)	73.85 ± 4.06	85.61 ± 13.53	102.64 ± 12.67	90.13 ± 7.13	**None**
Particulate COX-2 (arbitrary units)	186.77 ± 18.30	225.53 ± 23.80	11.82 ± 4.12	11.12 ± 2.91	**Genotype** ($P < 0.0001$)

[1] Data is presented as mean ± SEM. (n = 8-9)
[2] Numbers in rows that contain different superscripts are significantly different.

Table 7. Thromboxane A_2 in kidneys (endogenous levels), in 60 min incubations (*in vitro* steady-state levels) and produced by renal cyclooxygenase activities in Han:SPRD-*cy* rats consuming casein compared to soy protein based diet

Genotype	Normal		PKD		Effects
Diet	**Casein**	**Soy Protein**	**Casein**	**Soy Protein**	
Endogenous level (ng/mg protein)	0.26 ± 0.02[1]	0.26 ± 0.02	1.26 ± 0.07	1.12 ± 0.08	**Genotype** ($P < 0.0001$)
Steady-state level (ng/mg protein)	$0.46^{a,2}$ ± 0.06	0.47^{a} ± 0.02	6.12^{c} ± 0.92	3.11^{b} ± 0.49	**Geno × Diet**
Total COX activity (ng/min/mg protein)	0.04^{a} ± 0.01	0.03^{a} ± 0.00	0.38^{c} ± 0.05	0.20^{b} ± 0.04	**Geno × Diet**
COX-1 activity (ng/min/mg protein)	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	**Genotype** ($P = 0.0004$)
COX-2 activity (ng/min/mg protein)	0.03^{a} ± 0.01	0.03^{a} ± 0.00	0.32^{c} ± 0.04	0.17^{b} ± 0.02	**Geno × Diet**

[1] Data is presented as mean ± SEM. (n = 8-9)
[2] Numbers in rows that contain different superscripts are significantly different.

Table 8. Prostaglandin E_2 in kidneys (endogenous levels), in 60 min incubations (*in vitro* steady-state levels) and produced by renal cyclooxygenase activities in Han:SPRD-*cy* rats consuming casein compared to soy protein based diet

Genotype	Normal		PKD		Effects
Diet	**Casein**	**Soy Protein**	**Casein**	**Soy Protein**	
Endogenous level (ng/mg protein)	1.34 ± 0.34[1]	1.17 ± 0.12	2.72 ± 0.54	2.00 ± 0.21	**Genotype** (P = 0.0003)
Steady-state level (ng/mg protein)	2.66 ± 0.34	2.53 ± 0.21	6.75 ± 1.31	5.14 ± 0.51	**Genotype** (P < 0.0001)
Total COX activity (ng/min/mg protein)	0.13 ± 0.02	0.11 ± 0.01	0.28 ± 0.05	0.22 ± 0.02	**Genotype** (P < 0.0001)
COX-1 activity (ng/min/mg protein)	0.01 ± 0.01	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.02	**None**
COX-2 activity (ng/min/mg protein)	0.11 ± 0.02	0.10 ± 0.01	0.23 ± 0.03	0.21 ± 0.03	**Genotype** (P < 0.0001)

[1] Data is presented as mean ± SEM. (n = 8-9)

Table 9. Prostaglandin I_2 in kidneys (endogenous levels), in 60 min incubations (*in vitro* steady-state levels) and produced by renal cyclooxygenase activities in Han:SPRD-*cy* rats consuming casein compared to soy protein based diet

Genotype	Normal		PKD		Effects
Diet	**Casein**	**Soy Protein**	**Casein**	**Soy Protein**	
Endogenous level (ng/mg protein)	1.87 ± 0.25[1]	1.88 ± 0.07	7.37 ± 0.68	6.71 ± 0.55	**Genotype** ($P < 0.0001$)
Steady-state level (ng/mg protein)	8.21 ± 0.98	6.91 ± 0.37	41.89 ± 4.49	31.80 ± 4.13	**Genotype** ($P < 0.0001$)
Total COX activity (ng/min/mg protein)	$0.29^{a,2}$ ± 0.04	0.28^{a} ± 0.03	2.11^{c} ± 0.24	1.18^{b} ± 0.17	**Geno × Diet**
COX-1 activity (ng/min/mg protein)	$0.03^{a,2}$ ± 0.01	0.00^{a} ± 0.02	0.25^{b} ± 0.05	0.06^{a} ± 0.06	**Geno × Diet**
COX-2 activity (ng/min/mg protein)	$0.26^{a,2}$ ± 0.03	0.28^{a} ± 0.02	1.61^{c} ± 0.19	1.04^{b} ± 0.13	**Geno × Diet**

[1] Data is presented as mean ± SEM. (n = 8-9)
[2] Numbers in rows that contain different superscripts are significantly different.

Table 10. Total prostanoids in kidneys (endogenous levels), in 60 min incubations (*in vitro* steady-state levels) and produced by renal cyclooxygenase activities in Han:SPRD-*cy* rats consuming casein compared to soy protein based diet

Genotype	Normal		PKD		Effects
Diet	**Casein**	**Soy Protein**	**Casein**	**Soy Protein**	
Endogenous level (ng/mg protein)	3.72 ± 0.72[1]	3.36 ± 0.22	11.40 ± 1.19	9.82 ± 0.77	**Genotype** ($P < 0.0001$)
Steady-state level (ng/mg protein)	10.43 ± 1.07	9.91 ± 0.38	56.29 ± 5.98	40.05 ± 4.64	**Genotype** ($P < 0.0001$)
Total COX activity (ng/min/mg protein)	$0.44^{a,2}$ ± 0.06	0.43^{a} ± 0.04	2.68^{c} ± 0.33	1.61^{b} ± 0.21	**Geno × Diet**
COX-1 activity (ng/min/mg protein)	0.04 ± 0.01	0.01 ± 0.01	0.34 ± 0.06	0.25 ± 0.13	**Genotype** ($P = 0.0010$)
COX-2 activity (ng/min/mg protein)	$0.39^{a,2}$ ± 0.05	0.42^{a} ± 0.03	2.21^{c} ± 0.27	1.42^{b} ± 0.16	**Geno × Diet**

[1] Data is presented as mean ± SEM. (n = 8-9)
[2] Numbers in rows that contain different superscripts are significantly different.

Dietary soy protein intervention selectively reduced the higher COX activities in diseased kidneys (Tables 7-10). COX-1 activity only was significantly lower (by 76%) in diseased kidneys from soy fed compared to casein fed rats when determined by production of the major prostanoid, namely PGI_2. COX-2, as well as total COX activity were 40-50% lower in kidneys from soy fed compared to casein fed rats when measured by TXA_2, PGI_2 or total prostanoid production. When either COX-1 or COX-2 activity was measured by PGE_2 production, there was no significant effect of the soy protein diet on these COX activities (Table 8).

In order to determine whether the relative proportions of renal prostanoids were altered by disease, ratios were compared. The higher ratios of TXA_2/PGE_2 (Figure 2) and TXA_2/PGI_2 (Figure 3) demonstrate that the change in TXA_2 is greater than either PGE_2 or PGI_2 in diseased kidneys compared to normals. The higher ratios of PGI_2/PGE_2 (Figure 4) demonstrate that the change in PGI_2 is greater than PGE_2 in diseased kidneys compared to normals. Hence, the relative change in prostanoid levels in disease was in the order of $TXA_2 > PGI_2 > PGE_2$.

Soy protein feeding did not alter the relative proportions of endogenous and *in vitro* steady-state levels of prostanoids as these ratios were not altered by diet. The ratios of COX-2 activities determined by production of prostanoids, however, were altered by dietary soy protein intervention. The COX-2 activity ratios for TXA_2/PGE_2, TXA_2/PGI_2, and PGI_2/PGE_2 were ~25-35% lower in kidneys from soy fed compared to casein fed rats. Hence, the effect of soy protein on COX-2 activity was in the order of $TXA_2 > PGI_2 > PGE_2$ produced.

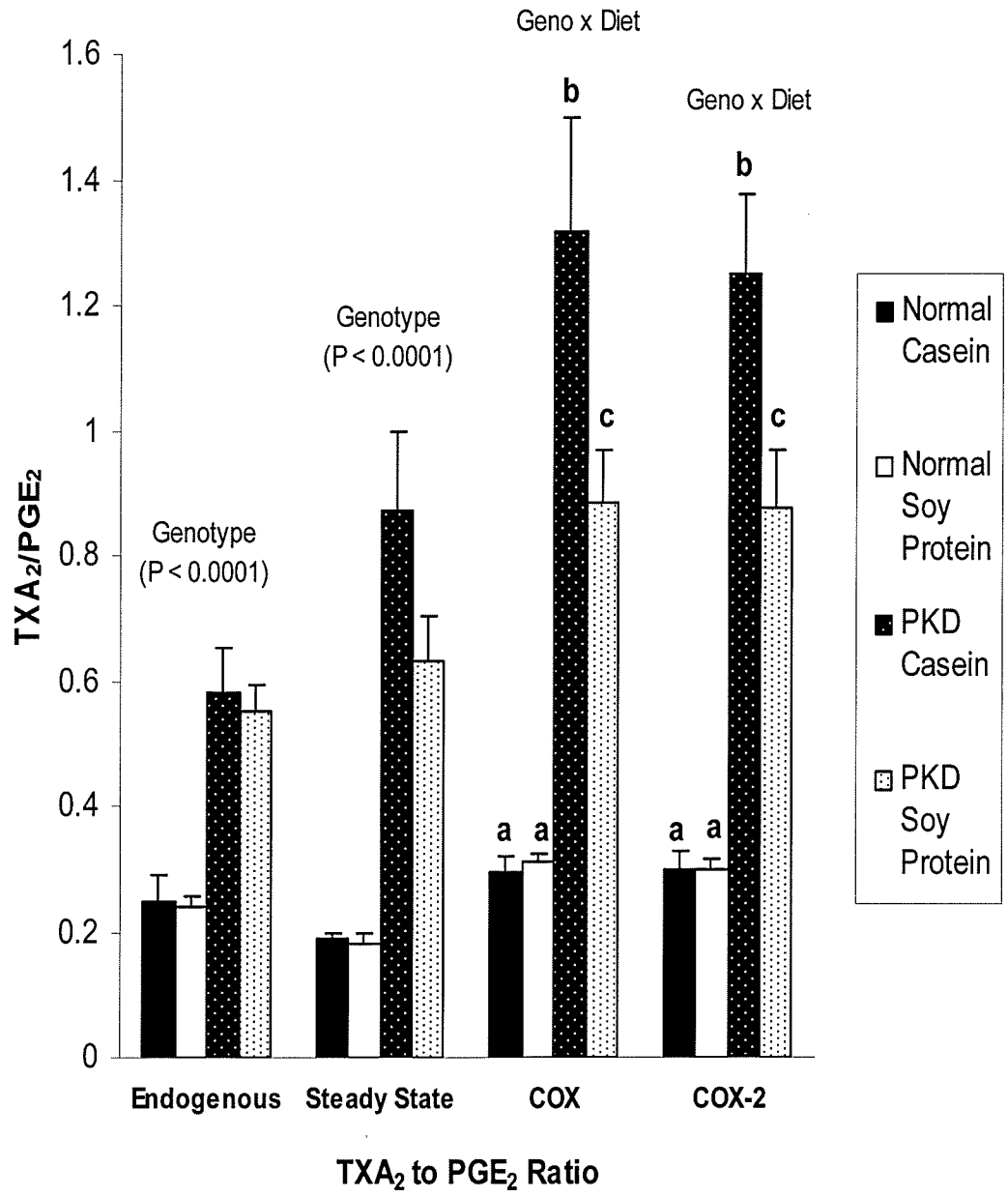


Figure 2. Ratio of TXA_2 to PGE_2 in kidneys (endogenous levels), in 60 min incubations (*in vitro* steady-state levels) and produced by renal cyclooxygenase activities in Han:SPRD-*cy* rats.
Values with different letters within each assay condition are significantly different ($P<0.05$).

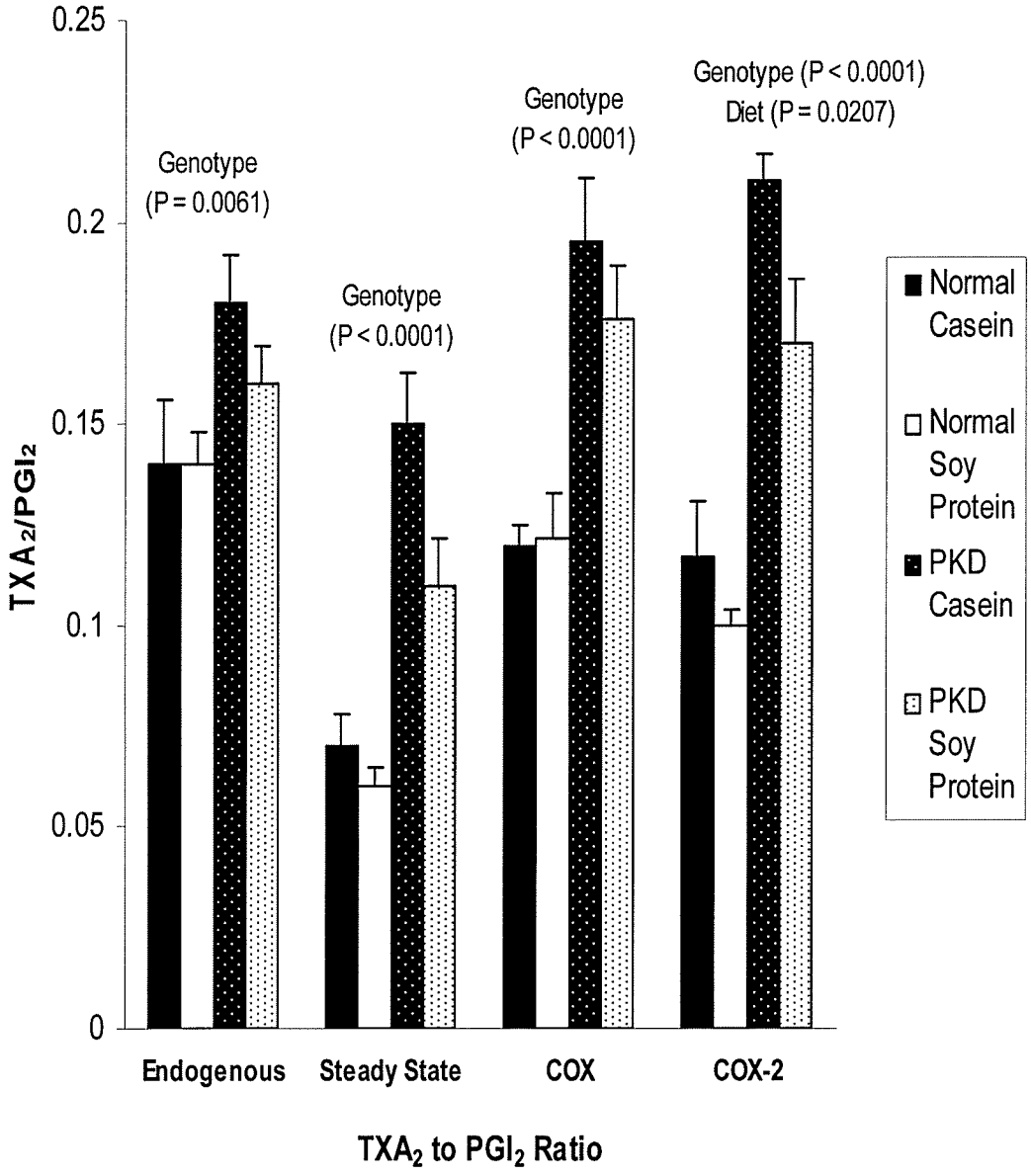


Figure 3. Ratio of TXA_2 to PGI_2 in kidneys (endogenous levels), in 60 min incubations (*in vitro* steady-state levels) and produced by renal cyclooxygenase activities in Han:SPRD-*cy* rats.

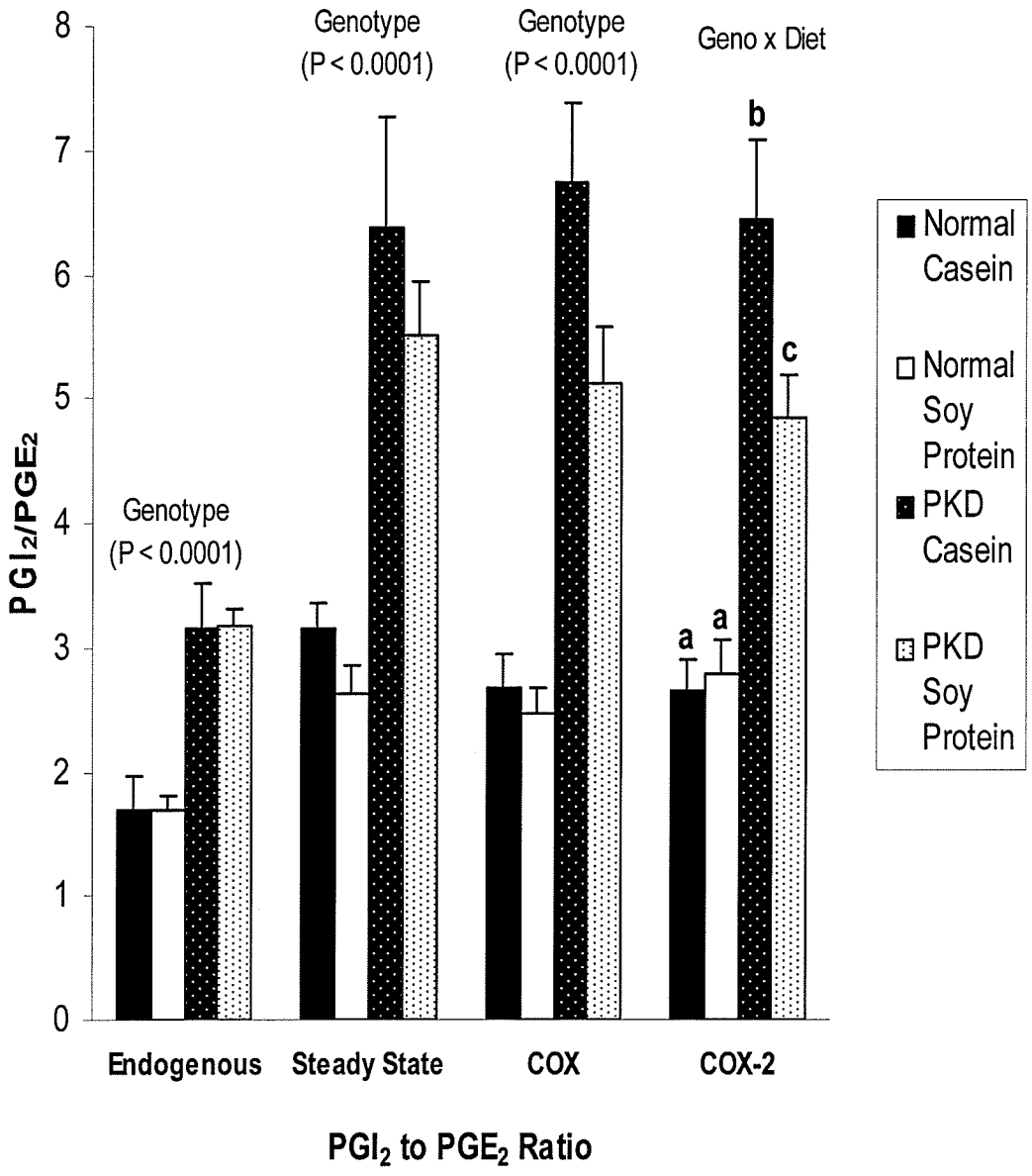


Figure 4. Ratio of PGI_2 to PGE_2 in kidneys (endogenous levels), in 60 min incubations (*in vitro* steady-state levels) and produced by renal cyclooxygenase activities in Han:SPRD-*cy* rats.
Values with different letters within each assay condition are significantly different ($P<0.05$).

DISCUSSION

These results demonstrate that both disease and dietary soy protein intervention have specific effects on COX isoform activities and renal prostanoid production. Dietary soy protein attenuated the higher protein levels of particulate $cPLA_2$ in the diseased kidneys. This suggests less active $cPLA_2$ is available for releasing AA from cell membranes resulting in less substrate for COX activity. Renal COX activities and production of TXA_2, PGE_2 and PGI_2 were significantly elevated in disease in Han:SPRD-*cy* rats. Increased COX-1 activity in diseased kidneys resulted in increased production of TXA_2 and PGI_2 while increased COX-2 activity resulted in higher production of all three prostanoids. The effect of dietary soy protein on prostanoid production and COX activities in diseased kidney also was selective. Soy protein reduced *in vitro* steady-state levels of TXA_2, but not the other two prostanoids. Soy protein feeding attenuated COX activity producing TXA_2 more than COX activities producing PGE_2 or PGI_2. The only COX-1 activity significantly impacted by soy protein was the COX-1 activity that produced PGI_2, while COX-2 activity that produced both PGI_2 and TXA_2 was attenuated by soy protein in diseased kidneys. In contrast, soy protein feeding did not alter any COX activities that produced PGE_2.

In the diseased kidneys the relative increase in TXA_2 is the greatest among the three prostanoids. TXA_2, the vasoconstricting prostanoid, is synthesized in the glomeruli [52,53], and is increased in conditions in which GFR is reduced such as kidney transplant rejection, hepatorenal syndrome and systemic lupus erythematosus [56,69]. Selective inhibition of TXA_2 synthesis is associated with an increased GFR, improved renal function and prevented renal histologic damage in experimental glomerulonephritis and

CHF [54,69,89]. In the current study, soy protein feeding specifically lowered the *in vitro* steady-state levels of TXA_2; hence, suggesting the improved renal function and delayed disease progression observed in diseased soy protein fed rats was associated with a selective decrease in renal TXA_2. Moreover, dietary soy protein lowered COX-2 activity, but not COX-1 activity producing TXA_2. This may reflect the fact that the major COX isoform activity producing TXA_2 in the diseased kidneys is COX-2 (about 85%) and therefore, is also the COX isoform that is significantly influenced by dietary soy protein intervention.

In contrast to TXA_2, prostaglandins are vasodilators, with PGI_2 being the major prostaglandin synthesized in glomeruli [52] and in whole kidney homogenates in the current study. The beneficial effects of increased renal prostaglandin production have been demonstrated in chronic glomerulonephritis and CHF [54,69]. In polycystic kidneys, enlarged renal cysts compromise renal function, alter intrarenal circulation in renal ischemia areas and activate the renin-angiotensin system [14]. Hence, the increased renal prostaglandin synthesis in diseased Han:SPRD-*cy* rats observed herein may elevate the decreased GFR, counteract the vasoconstriction caused by angiotensin as well as TXA_2, and contribute to renal homeostasis. Dietary soy protein intervention did not change the endogenous levels of prostaglandins; therefore, these beneficial effects of prostaglandins were maintained, while TXA_2 levels were lower in soy protein fed rats.

The specific pattern of distribution of prostaglandins and COX isoforms may help to explain the effects of disease and dietary soy protein on these renal functional changes. COX-1 is found in collecting tubules [68] and is required for the full expression of natriuresis [73,90]. When increased prostaglandins increase GFR, cause natriuresis, and

result in the solute concentration in the renal filtrate to fall below a certain level, the epithelial cells of macula densa signal the juxtaglomerular cells to secrete renin to increase blood pressure and blood solute concentration [91]. COX-2 is detected in macula densa [68], and COX-2 inhibition prevents the increase in renal renin mRNA in animals fed a low-salt diet [92]. In current study, dietary soy protein decreased the elevated COX-1 and COX-2 activity producing PGI_2 in diseased kidneys, this may reduce natriuresis caused by increased prostaglandin production and further prevent the increase in renin and maintain renal fluid-electrolyte homeostasis.

Previous studies also demonstrate that dietary soy protein ameliorates disease progression by reducing renal epithelial cell proliferation and apoptosis, cyst growth and renal fibrosis [48,49,82]. In cultured mesangial cells, PGE_2 and PGI_2 inhibit cell proliferation and extracellular matrix deposition, while TXA_2 stimulates cell proliferation and mesangial cell matrix production [93-97]. Therefore, in the diseased kidneys, it appears that the effect of prostaglandins is not enough to prevent the proliferative effects of TXA_2 as well as other growth factors, such as epidermal growth factor (EGF) and IGF [83,98]. The decrease in TXA_2 production in diseased soy-fed rats may contribute to the reduced renal epithelial cell proliferation, apoptosis, renal cysts and renal fibrosis.

In a short term study which introduced dietary soy protein to weanling Han:SPRD-*cy* rats after as little as 1-3 weeks of feeding also showed that soy protein fed rats had reduced cyst growth and renal fibrosis [51]. *Ex vivo* production of PGE_2 from diseased compared with normal kidneys was lower in rats at this early stage of disease. Dietary soy protein compared with casein was reported to increase PGE_2 levels in diseased kidneys at both 1 and 3 wk of feeding. The elevated PGE_2 production in diseased soy protein fed

rats may help maintain normal renal hemodynamics in the face of cyst growth and fibrosis in early disease. Interestingly, no effects of soy protein feeding on PGE_2 production were observed later in disease or in normals in the current study. There may be several explanations which explain this apparent difference. First, disease and dietary soy protein may affect renal prostanoid production differently during different stages of the disease. Second, in this short time study, the PGE_2 production was based on net weight, since polycystic kidneys contain more water; it is difficult to determine whether the reduced renal PGE_2 production was caused by a reduction in renal tissue or by the disease itself. Moreover, in this short term study, renal production of PGI_2 and TXA_2 were not measured; therefore, the overall effect of soy protein in the early stages is still not clear.

Which specific compound(s) present in soy protein contributes to these beneficial effects is not understood. The phosphoinositides are a class of phospholipids involved in intracellular signal transduction, and tyrosine phosphorylation is important in renal phosphoinositide metabolism [99,100]. In DBA/2FG-*pcy/pcy* mice, this intracellular signalling pathway is altered in diseased kidneys [100,101]. Inhibition of epidermal growth factor receptor tyrosine kinase activity has been shown to attenuate disease progression in animal model of PKD [98,102,103]. In addition, abnormalities in growth factors, including IGF-1, have also been documented in animal models and in human PKD [83], and dietary soy protein intervention lowered renal IGF-1 levels in the Han:SPRD-*cy* rat [83]. Soy isoflavones, such as genistein can inhibit tyrosine kinase activity associated with cytokine and growth factor activation, and inhibit the proliferation of inflammatory cells [104]. This may explain the reduced renal

inflammation and cell proliferation observed in diseased rats in the current study that consumed soy protein diet. However, one previous study suggested that the beneficial effects of soy are not related to its genistein content [49]. Soyasaponin extracts on the other hand, impede kidney enlargement and cyst growth in the *pcy* mouse model of ADPKD [105]. It is suggested that soyasaponin may act as cell volume regulators, increase the open probability of the outwardly rectifying potassium channels to limit potassium accumulation and solute secretion within the cell and/or cysts. However, more studies are needed to fully investigate the beneficial components in soy and the mechanisms of action.

The amino acid composition of soy protein may also explain the beneficial effects. Compared with casein, soy protein contains higher level of L-arginine. L-Arginine is a precursor for a number of molecules involved in tissue injury and repair, such as nitric oxide (NO), polyamines, L-proline and agmatine [50,106]. Polyamines are essential for cell proliferation; L-proline is a key collagen constituent and is important for matrix build up; agmatine is a regulator of renal hemodynamics and glomerular filtration, and inhibits the key enzyme in polyamine production and the inducible nitric oxide synthase (iNOS) [106]. When iNOS is induced, large, cytotoxic quantities of NO are generated [106]. Studies using therapeutic dietary L-arginine supplementation in renal disease have shown inconsistent effects. High L-arginine intake has been shown to reduce the severity of hypertensive renal diseases presumably by enhancing the deficient endothelial NO production, which is a critical mediator of vasodilation [106]. In other renal diseases, such as acute anti-thy-1 glomerulonephritis and experimental lupus nephritis, L-arginine supplementation showed deleterious effects, and it may be due to the induction of iNOS.

In *pcy* mice, adding L-arginine to a low protein diet for 3 months does not affect renal size (our unpublished observations). Therefore, whether L-arginine is the amino acid that contributes to the beneficial effects of soy protein intervention in the Han:SPRD-*cy* rats with renal disease needs further investigation.

The finding that animal models of ADPKD have altered levels and activities of COX-1 and COX-2 adds to the questions regarding treatment of renal disease patients with non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors. NSAIDs are non-selective COX inhibitors which inhibit prostanoid production and reduce inflammation [53,64]. However, they also can result in adverse side effects such as gastrointestinal ulcerations and renal effects such as decreased renal blood flow and GFR [53,73]. These side effects are believed to be due to the inhibition of the COX-1, which generates the cytoprotective and renoprotective prostanoids, such as PGE_2 and PGI_2 [64,73]. COX-2 selective inhibitors were designed to inhibit the production of COX-2 dependent inflammatory prostanoids and leave the cytoprotective COX-1 products [63,64,73], but there is still controversy about whether inhibition of COX-2 is protective or deleterious in renal disease. Studies show that using the selective COX-2 inhibitor, NS398 reduces proteinuria in rat model of 5/6 renal ablation [107]; flosulide, also reduces proteinuria in a rat model of passive Heymann nephritis [108]. Etodolac, another COX-2 inhibitor, decreases fibrosis and tubular apoptosis in a rat model of unilateral ureteral obstruction [109]. In contrast to the above studies indicating a beneficial effect of COX-2 inhibition, a selective COX-2 inhibitor was shown to impair glomerular capillary healing, enhance glomerular monocyte chemoattractant protein-1 formation and monocyte recruitment in experimental glomerulonephritis [110,111].

Constitutive expression of COX-2 is essential for normal kidney development [63,74], and the protein levels of COX-2 in polycystic kidneys in Han:SPRD-cy rats are already substantially lower than that of normals, even though the activities are increased by disease. Future studies are required to examine if it is safe to use COX-2 selective inhibitors in ADPKD patients. Significantly however, results from the current study indicates that dietary soy protein may be a safe way to treat PKD since it not only delays disease progression, preserves renal function but also decreases COX activities and reduces renal inflammation in Han:SPRD-*cy* rats.

In summary, our study demonstrated that prostanoid production and COX isoforms are selectively altered by renal disease and dietary soy protein intervention in the Han:SPRD-*cy* rat model of ADPKD. The beneficial effects of dietary soy protein in renal disease are associated with selective effects on prostanoid production and COX isoform activities. However, our study did not demonstrate whether the effect of soy protein on prostanoids is a result of changing disease or a direct effect on prostanoids; future studies are required to answer this question. In addition, human studies also are required to determine whether ADPKD patients could benefit from consuming soy protein as has been observed in this animal model.

3. DISCUSSION

3.1 Overall Discussion

PKD is the most commonly inherited renal disease, affecting 600,000 US citizens and an estimated 12.5 million people worldwide [1,2]. It accounts for 8-10% of the cases of ESRD and costs for dialysis and transplantation exceed more than $1.5 billion annually in the United States [1,3,4]. There is still no cure for PKD. In the weanling Han:SPRD-*cy* rat model of ADPKD, dietary soy protein intervention delays disease progression after consuming the diet for as little as 1-3 to 8 weeks [48,49,50,51,82,83]. Since ADPKD begins as early as in *utero* and progresses slowly through the growing stages and into adulthood, and maternal diet influences the fetus as well as milk composition [85,86], it was reasonable to assume that dietary soy protein intervention in pre-weaning stages will delay disease progression in the Han:SPRD-*cy* rat model of ADPKD. However, the effects of initiating dietary soy protein intervention in *utero* and during lactation by manipulating the maternal diet have never been studied before.

The kidney is a rich source of prostanoids which regulate renal hemodynamics, water and solute transport, and renin secretion [52]. Renal prostanoid production has been shown to be altered by diseases and dietary interventions [53,54,72,79,80]. Altered renal protein levels of $cPLA_2$ and both COX-1 and COX-2 isoforms are detected in diseased Han:SPRD-*cy* rats [76]. However, the effect of disease and dietary soy protein on prostanoid biosynthetic enzymes and renal prostanoid production is not known.

The objectives of this study were to examine the effects of dietary soy protein in the maternal diet (initiated before conception, during pregnancy and lactation) on progression of renal injury in the Han:SPRD-*cy* rat model of ADPKD, and to examine the effects of

disease and dietary soy protein intervention on renal prostanoid biosynthetic enzymes and renal prostanoid production.

The hypotheses of this study were that:

(1) Dietary soy protein intervention will delay progression of renal injury in the offspring of Han:SPRD-*cy* rat model of ADPKD if it is initiated in the maternal diet.

(2) The beneficial effects of dietary soy protein intervention in both the maternal and post-weaning diet are associated with the alterations in the protein levels of renal prostanoid biosynthetic enzymes, namely, $cPLA_2$, COX-1 and COX-2.

(3) Disease increases renal PGE_2, PGI_2 and TXA_2 production and selectively increases COX-1 activity.

(4) Soy protein in the post-weaning diet selectively decreases renal TXA_2 production and COX-1 activity.

Our first hypothesis was that dietary soy protein intervention will delay progression of renal injury in the offspring of Han:SPRD-*cy* rat model of ADPKD if it is initiated in the maternal diet. The results not only confirm that dietary soy protein in the post-weaning diet delays renal disease progression, but also demonstrate that dietary soy protein in the maternal diet provides further benefits in offspring of the Han:SPRD-*cy* rats. Consistent with previous studies [48,83], soy protein compared to casein in the post-weaning diet resulted in higher urine creatinine, lower serum creatinine, higher creatinine clearance, lower serum urea nitrogen, lower left kidney weight relative to body weight, lower cyst scores and reduced renal inflammation. Compared to other dietary interventions that have been used to delay disease progression in ADPKD, such as

protein restriction [39], dietary CLA intervention [26,27] and dietary flaxseed intervention [21], dietary soy protein intervention in the post-weaning diet is as effective or better than other interventions since it delays as many or more markers of renal injury and renal function. Soy protein in the maternal diet also independently reduced renal inflammation and proteinuria. In addition, soy protein in either the maternal or post-weaning diet reduced renal cell proliferation, with either intervention being equally effective (Tables 3,4).

No difference was found with respect to final body weight and food and water intake between diseased rats that consumed casein or soy protein in the maternal and/or post-weaning diets (Table 2). Therefore, the beneficial effect observed in our study is not due to the impaired growth caused by reduced protein intake. The true digestibilities of casein and soy protein are high (97 to 98%), so N intake on these two diets is similar [112]. In addition, the soy protein is heat treated so that soybean trypsin inhibitor enzyme would be inactivated [99]. Since both casein and soy protein are low in sulfur amino acids, the AIN-93G diet that we used in our study also includes supplementation with sulfur amino acids [87]. The lipid source in the AIN-93G diet is soybean oil [87]; however, corn oil was used in the current study. Corn oil contains high levels of linoleic acid, but very little amount of linolenic acid. Since linoleic acid is the precursor of AA which is the precursor of the series 2 prostanoids, and linolenic acid is the precursor of the series 3 prostanoids [59], using corn oil instead of soybean oil can help to avoid the cross-reactivity with the series 3 prostanoids when we measure renal prostanoid production. However, some studies show that prolonged linolenic acid deficiency may result in visual problems and changes in learning behaviours [57].

Normal proliferation of renal tubular epithelial cells ceases before birth, but in ADPKD, cystic epithelia proliferate abnormally throughout life [9]. Moreover, cultured ADPKD epithelial cells have increased capacity for proliferation and survival [9]. Soy isoflavones, such as genistein can inhibit tyrosine kinase activity, which is associated with cytokine and growth factor activation, and inhibit the cell proliferation [104]. This may explain the reduced proliferation of renal cells and inflammatory cells observed in the diseased rats consuming soy protein in the maternal and/or post-weaning diet. However, one previous study showed that the beneficial effects of soy are not related to its genistein content [49]. In addition, renal cyst development is minimal by three weeks of age and is more prominent by eight to ten weeks of age; renal fibrosis is seen as early as two weeks of age and is increased in later stages of the disease in Han:SPRD-*cy* rats [5]. Soy protein in the maternal diet did not significantly affect cyst growth, renal fibrosis and renal function, this may be due to the fact that clinical symptoms and abnormal renal function are usually not present before birth; therefore, the effects of dietary soy protein in the maternal diet were not detected. Even though the effects of dietary soy protein in the maternal diet is not as prominent as it is in the post-weaning diet, the results do support our hypothesis that dietary soy protein intervention delays progression of renal injury in the Han:SPRD-*cy* rat model of ADPKD when it is initiated before conception, during pregnancy and lactation.

Our second hypothesis was that the beneficial effects of dietary soy protein intervention in both the maternal and post-weaning diet are associated with the alterations in the protein levels of renal prostanoid biosynthetic enzymes, namely, $cPLA_2$, COX-1 and COX-2. Our data not only confirm that renal protein levels of $cPLA_2$ in the cytosolic

and particulate fractions and COX-1 were significantly elevated while COX-2 was significantly decreased in diseased compared to normal rats, but also demonstrate that dietary soy protein in the post-weaning diet compared to casein result in lower protein levels of $cPLA_2$ in the particulate fraction of diseased kidneys. On the other hand, dietary soy protein in the maternal diet did not alter the protein levels of any prostanoid biosynthetic enzymes (Table 5). The lower levels of $cPLA_2$ in the particulate fraction of diseased kidneys given dietary soy protein in the post-weaning diet indicates that less activated $cPLA_2$ is available for releasing AA from cell membranes, resulting in less substrate for COX isoforms to produce prostanoids. Therefore, the beneficial effects of dietary soy protein in the post-weaning diet are associated with the alterations in the protein levels of renal $cPLA_2$ in the particulate fraction, while the beneficial effects of dietary soy protein in the maternal diet are not associated with alterations of the protein levels of the COX enzymes.

Our third hypothesis was that in the Han:SPRD-*cy* rat model of ADPKD, disease increases renal PGE_2, PGI_2 and TXA_2 production and selectively increases COX-1 activity. The results show that renal TXA_2, PGE_2 and PGI_2 were all significantly elevated in diseased Han:SPRD-*cy* rats (Tables 6-9). Prostaglandins cause vasodilation and TXA_2 causes vasoconstriction [52], and in cultured mesangial cells, prostaglandins inhibit cell proliferation and extracellular matrix deposition while TXA_2 stimulates cell proliferation and extracellular matrix production [93-97]. It appears that in the diseased rats, vasodilation caused by increased prostaglandins is not enough to counteract the vasoconstriction caused by angiotensin and TXA_2; therefore GFR is decreased. Moreover, the effect of prostaglandins is not enough to prevent the proliferative effects of

TXA_2 as well as other growth factors, such as EGF and IGF [83,98]; therefore, cell proliferation, renal cysts and fibrosis are increased. Most importantly, our data demonstrate that even though COX-1 activity producing increased production of TXA_2 and PGI_2 was elevated, COX-2 activity was the predominant COX activity in both normal and diseased kidneys and produced all three prostanoids. Therefore, the results support the hypothesis that disease increases renal PGE_2, PGI_2 and TXA_2 production. However, the presence of disease not only increases COX-1 activity, but also COX-2 activity. In fact, the COX-2 activity contributes most of the COX activity in both normal and diseased kidneys.

Our last hypothesis was that in diseased kidneys, soy protein in the post-weaning diet selectively decreases renal TXA_2 production and COX-1 activity. The results show that in diseased kidneys, soy protein in the post-weaning diet selectively reduced *in vitro* steady-state levels of TXA_2, but not the other two prostanoids (Tables 6-9). The improved renal function and delayed disease progression observed in the diseased rats that consumed soy protein in the post-weaning diet may be associated with a selective decrease in renal TXA_2 and the maintenance of the beneficial effects of prostaglandins. More importantly, soy protein in the post-weaning diet not only reduced COX-1 activity that produced PGI_2 but also reduced COX-2 activity that produced both PGI_2 and TXA_2. The reduction in the elevated COX-1 and COX-2 activity producing PGI_2 in diseased kidneys may reduce natriuresis caused by increased prostaglandin production and further prevent the increase in renin and maintain renal fluid-electrolyte homeostasis. Therefore, the results support the hypothesis that in diseased kidneys, soy protein in the post-

weaning diet selectively decreases renal TXA_2 production but do not support the hypothesis that soy protein in the post-weaning diet selectively decreases COX-1 activity.

In summary, our study is the first to demonstrate that dietary soy protein in the maternal diet provides further benefits in offspring with renal disease, and that the beneficial effects of dietary soy protein in the post-weaning diet are associated with select effects on COX isoforms and renal prostanoid production. Human studies are required to determine whether ADPKD patients would benefit from consuming soy protein as has been observed in this study and if the beneficial effects are also associated with the alterations in COX isoforms and renal prostanoid production.

3.2 Strengths and Limitations

One strength of this study is that this is the first study demonstrating the beneficial effects of initiating dietary soy protein intervention in *utero* and during lactation by manipulating the maternal diet. The beneficial effects observed in our study provide a potential approach to have more of an impact on disease progression if dietary soy protein intervention is started earlier. If soy protein intervention is introduced both in the maternal and post-weaning diet, renal inflammation would be reduced even more.

Another strength of this study is that this is the first study investigating the effects of dietary soy protein on renal prostanoid production and prostanoid biosynthetic enzymes, namely $cPLA_2$, COX-1 and COX-2.

One limitation of this study is that the soy protein isolate we used in our study is not a pure protein. A range of biologically active compounds contained in the soy protein isolate may contribute to the beneficial effects observed in our study. We are not able to

determine if the beneficial effects of soy protein intervention observed in our study are caused by these biologically active compounds or by soy protein itself.

Another limitation of this study is that individual prostanoid and COX isoforms are not homogenously present in the kidney, but are synthesized within distinct anatomic locations. In our study, we did not measure individual prostanoid and COX isoforms by location.

Also, our study did not demonstrate whether the effect of soy protein intervention on prostanoid production is a cause or effect of the altered disease progression. We are not able to determine whether the decreased COX activities and renal prostanoid production are a result of the delayed disease progression or are the cause of preserved renal function and ameliorated renal injury.

3.3 Directions for Future Research

To further elucidate how dietary soy protein slows disease progression of renal injury in the Han:SPRD-*cy* rat model of ADPKD, we have several directions to follow:

1. Measure renal individual prostanoid, COX-1 and COX-2 enzymes by location. Since renal prostanoids and COX isoforms are not homogenously present in the kidney, understanding their specific distribution and the changes caused by dietary interventions will help to better explain how dietary intervention benefits diseased kidneys.
2. Measure the activities of $cPLA_2$. The protein levels of $cPLA_2$ are altered by both disease and soy protein intervention. However, the activitiy of $cPLA_2$ is not known. Measuring the activities of $cPLA_2$ will help us to better understand the

relationship between the changes in $cPLA_2$ and the changes in prostanoid production.

3. Measure the eicosanoids produced from the lipoxygenase and cytochrome *P*-450 pathways. Metabolites of these two pathways are also involved in inflammation and renal hemodynamic action. Measuring the metabolites from these two pathways will help to fully clarify the effects of disease and dietary interventions on renal functional and histological changes.
4. Investigate whether the beneficial effects we observed in our study are due to certain soy components, such as soyasaponin, diadzein and glycetein. These compounds are thought to have beneficial biological effects, but further studies are needed to determine if they are the components that contribute to the beneficial effects observed in our study and to determine their most effective dose and mechanism of action.

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5. APPENDIX

5.1 Measurement of Steady-State Protein Levels of $cPLA_2$, COX-1 and COX-2

5.1.1 Lyophilization of Kidneys

Each right kidney was lyophilized in preparation for western immunoblotting of steady-state protein levels of $cPLA_2$, COX-1 and COX-2. Frozen kidneys were weighed, cut into small pieces, and placed in pre-weighed 15mL tubes with lids that have holes, immersed in liquid nitrogen and then placed in the freeze dryer (Freeze Dry System, Labconco, Kansas City, Missouri). Tissue samples were considered dry when two consecutive equal weights were obtained. Dried kidneys were pulverized in the tubes using a spatula. The lids were replaced with ones without holes and the samples were store at $-80^{\circ}C$ till homogenization.

5.1.2 Homogenization of Kidneys

The lyophilized kidneys were pulverized and thoroughly mixed so that a representative sample of the kidney could be taken. Twenty milligrams of pulverized kidneys were weighed out into labeled tubes, placed on ice and homogenized in 100 volumes of ice-cold cytosolic homogenization buffer twice, for 30 seconds each time using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario). All reagents used for homogenization were purchased from Sigma (St. Louis, Missouri), unless otherwise stated. Cytosolic homogenization buffer contained 500 mM Tris (Fisher Scientific, Mississauga, Ontario), 0.5 M sucrose, 200 mM ethylene-diamine-tetraacetic acid (EDTA) (pH=7.6), 100 mM ethylene glycol-bis (β-aminoethyle ether) N,N,N',N'-tetraacetic acid (EGTA) (pH=7.5), 0.4 M NaF, 10 mM sodium orthovanadate, 2.5 mg/mL

aprotinin, 1mg/mL leupeptin, 2.5 mg/mL pepstatin, 1 mg/mL soybean trypsin inhibitor (STI), 24 mg/mL 4-(2-aminoethyl) benzene-sulfonyl fluoride (ABSF), β-mercaptoethanol (Fisher Scientific, Mississauga, Ontario), and deionized water. Homogenates were transferred into 5mL tubes and ultra-centrifuged at 100,000 x *g* for 30 minutes at 4°C using the Beckman L5-50B ultracentrifuge (Mississauga, Ontario). The supernatant was removed, collected as the cytosolic fraction, and stored immediately at -80°C. The remaining pellet was re-suspended in 20 volumes of particulate homogenization buffer using a glass rod and the Vortex-Genie 2 (VWR Scientific Products, West Chester, Pennsylvania). Particulate homogenization buffer contained 1% triton X-100 in addition to the compounds present in the cytosolic homogenization buffer. Samples were stored on ice for 10 minutes and ultra-centrifuged again at 100,000 x *g* for 30 minutes at 4°C. The supernatant was removed, collected as the particulate fraction, and stored immediately at -80°C.

5.1.3 Microassay for Quantitation of Protein

Protein concentrations were determined by protein assay as described by Bradford [88]. Costar 96-well microplates (Conring Incorporation, Corning, New York) were used. Wells were labeled as blank, standard or sample, and standard concentrations of 0.05, 0.10, 0.20, 0.30, 0.04 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 loaded into the wells in triplicate. Two hundred μL of room temperature Bradford Reagent (Sigma, St. Louis, Missouri) was added to each well, and the plates were set on the orbital shaker (Fisher Scientific, Fair Lawn, New

Jersey, Model No. 361) for approximately 5 minutes until there was no precipitate. The plates were then incubated at room temperature for 10 minutes and 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 protein concentration of each sample was calculated according to its standard curve.

Protein Concentration (mg/mL) =

[(Concentration of standard × absorbance of sample)/ absorbance of standard] × 20

5.1.4 Western Immunoblotting

Steady-state levels of $cPLA_2$, COX-1 and COX-2 were measured by using one-dimensional sodium dodecyl sulphate (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 charge, size and shape determines the migration rate of each protein. $cPLA_2$ was found in the cytosolic and particulate fractions, while COX-1 and COX-2 were found in the particulate fraction only. Cytosolic fraction was treated with 6X sample buffer and particulate fraction was treated with 2X sample buffer due to the differences in the total protein concentration of the kidney homogenates and the total amount of protein required for analyzing the steady-state levels of the specific protein of interest. All reagents used for sample buffer were purchased from Fisher Scientific (Mississauga, Ontario). 8 mL 2X sample buffer contained 1.0 mL 0.5M Tris-HCl (pH 6.8), 0.8 mL glycerol, 1.6 mL 10% (w/v) SDS, 0.4 mL β-mercaptoethanol, 0.4 mL 1% (w/v)

bromophenol blue and 3.8 mL deionized water. To make 10 mL of 6X sample buffer, mix 3.5 mL 1M Tris-HCl (pH 6.8), 3 mL glycerol, 1g SDS, 0.6 mL β-mercaptoethanol, 0.5 mL 1% (w/v) bromophenol blue and fill to 10 mL with deionized water. After samples were prepared, they were heated at 90-100°C for 5 minutes to denature proteins, placed in the Eppendorf 5417C centrifuge (Brinkmann Instruments, Mississauga, Ontario) at 7000 rpm for 1 minute, and placed on ice.

Electrophoresis

A 7.5% separating gel was made and poured into the casting gel apparatus to 3/4 full and overlaid with a small amount of deionized water. 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. Ten mL of separating gel contained 5.34 mL deionized water, 2.5 mL 1.5M Tris-HCl (pH 8.8), 100 µL 10% (w/v) SDS, 2.0 mL acrylamide : bis-acrylamide 29:1 40% stock solution, 50 µL 10% (w/v) ammonium persulfate (APS) and 10 µL N,N,N',N'-tetramethylethylenediame (TEMED). 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 and 5 µL TEMED. All reagents used for the separating and stacking gels were purchased from Fisher Scientific (Mississauga, Ontario).

Once the stacking gel was solidified, the casting gel apparatus was placed in the electrophoretic chamber. MiniVE Vertical Electrophoresis System (Amersham Biosciences, Baie d'Urfé, Québec) was used. 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 1X running buffer, a 10X running buffer was first made

by mixing 29.0 g Tris base, 144.0 g glycine, 10.0 g SDS, and filling to 1L with deionized water. One hundred mL of this 10X running buffer was then diluted with 900 mL of deionized water to make 1X running buffer. All reagents used for running buffer were purchased from Fisher Scientific (Mississauga, Ontario). Each well was washed with 100 μL of running buffer and samples were loaded into each well. On each gel, two wells were loaded with 14 μL of standardized protein so that the optical density of each band produced could be calculated as a percentage of the standard; one well was loaded with 11 μL of molecular weight marker (BenchMark™ Pre-Stained Protein Ladder, Cat # 10748-010, Invitrogen Life Technologies, Carlsbad, California) to ensure that the protein band of interest was at its specific molecular weight after immunoblotting. The apparatus was then attached to a power source (EPS 301 Power Supply, Amersham Biosciences, Baie d'Urfé, Québec) and run at 200V at room temperature for 100 mins.

Assemble the Immunoblot Sandwich

The proteins that were embedded in the gel matrix can be transferred onto the surface of a membrane, thus providing access for reaction with the antibodies. After electrophoresis, proteins were transferred from the gel to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Baie d'Urfé, Québec). 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 s, then in water for 8 mins and finally in 1X transfer buffer for equilibration until the gel was ready for protein transfer. Before protein transfer, the gel was also equilibrated in 1X transfer buffer for 10 mins. 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. One hundred mL of this 10X transfer buffer was mixed with 200 mL of HPLC grade methanol and 700 mL of deionized water to make 1X transfer buffer. All reagents used for transfer buffer were purchased from Fisher Scientific (Mississauga, Ontario). The transfer apparatus was set up with the gels and the membranes placed in such a way as to form a "sandwich" consisting of one sponge, one piece of filter paper (Cat# 30306188, Whatman International Ltd., England), the gel, which is topped with the membrane, a piece of filter paper on top of the membrane and finally 8 more sponges on top of the filter paper. All of these were soaked in 1X transfer buffer.

Transfer Proteins From Gel to Membrane

The transfer apparatus was filled with 1X transfer buffer and placed in the transfer chamber which was also filled with 1X transfer buffer. Proteins were transferred for 2 hours at 375mA at 4°C. And the transferred proteins were bound to the surface of the membrane.

Immunoprobing with Specific Antibodies

Once the transfer was complete, the membrane was removed from the transfer apparatus and placed in 5% skim milk solution (prepared in TBS/Tween) on the rocker (Boekel Scientific Rocker II, model 260350, Feastville, Pennsylvania) at room temperature for one hour. This 5% skim milk solution was used to block non-specific protein-binding. 0.1% TBS/Tween solution was prepared by mixing 24.2 g Tris base with 80 g of NaCl and filling with deionized water to 800 mL. The pH was adjusted to 7.6 using HCl. Ten mL of enzyme grade Tween was added and deionized water was also

added to make up to 1000 mL. 100 mL of this solution was then diluted with 900 mL deionized water.

Primary antibody solution consisting of 2% skim milk solution which was prepared in 0.1% TBS/Tween was poured over the membrane and placed on a shaker in the 4°C refrigerator overnight. Following the treatment with the primary antibody, the membrane was washed with 0.1% TBS/Tween for a length of time specified by the type of protein to be detected. For specific condition of primary antibody, secondary antibody and washing time for each protein of interest please see Table 11. Secondary antibody that is conjugated to horseradish peroxidase was made in 0.1% TBS/Tween, poured over the membrane and set on the orbital shaker at room temperature for 1 hour. The membrane was then washed again with 0.1% 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.

Developing the Membrane

Detection solutions using ChemiGlow™ (Alpha Innotech Corporation, San Leandro, California), a chemiluminescent substrate for horseradish peroxidase, was mixed approximately 15 minutes before the membrane was ready to be developed and placed in a dark place until use. After the last wash, TBS/Tween was poured off the membrane and the membrane was placed on a piece of saran wrap. ChemiGlow™ solution was evenly distributed over the surface of the membrane and left to sit for 5 minutes. ChemiGlow™ solution was then drained off and the membrane was carefully placed in a new piece of saran wrap ensuring that all air bubbles were removed. The membrane was then developed using Fluorchem™ FC digital imaging system (Alpha Innotech Corporation,

San Leandro, California). For the time periods specified for each protein, please see Table 11. Once the bands were imaged, the optical densities (OD) of the bands were measured using the Fluorchem™ FC software. For each band, OD was determined by first calculating the integrated density value (IDV), which equals to the sum of each pixel values minus the background value. The average value (AVG) was then calculated by dividing the IDV by the size of the region in which the band is enclosed. 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.

Table 11. Western Immunoblotting: Summary of the specific conditions for each protein 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 Time (min)
Cytosolic $cPLA_2$ [a]	14	1:250 2% SMP	3 × 5 min	Anti-rabbit	1:20,000	3 × 5 min	1A:1B:10H_2O	30
Particulate $cPLA_2$ [a]	14	1:250 2% SMP	3 × 5 min	Anti-rabbit	1:20,000	3 × 5 min	1A:1B:10H_2O	30
Particulate COX-1 [b]	14	1:250 2% SMP	3 × 10 min	Anti-mouse	1:20,000	3 × 10 min	1A:1B:10H_2O	30
Particulate COX-2 [b]	14	1:250 2% SMP	3 × 10 min	Anti-rabbit	1:20,000	3 × 10 min	1A:1B:10H_2O	30

A = substrate A (ChemiGlo™ West Luminol/Enhancer Solution, Alpha Innotech Corporation, San Leandro, CA, USA)
B = substrate B (ChemiGlo™ West Stable Peroxide Solution, Alpha Innotech Corporation, San Leandro, CA, USA)
SMP = skim milk powder, supplied by Pacific instant skim milk powder, Vancouver, BC, Canada
[a] Supplied by Santa Cruz Biotechnology, Santa Cruz, California
[b] Supplied by Cayman Chemical, Ann Arbor, Michigan
[c] Supplied by Sigma, Saint Louis, Missouri