Effects of Conjugated Linoleic Acid Isomers on Eicosanoid Metabolism in Kidney and Liver Tissues of Obese Zucker Rats

by

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A Thesis submitted to the Faculty of Graduate Studies

of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Seventeen wk old male obese Zucker rats were given 0.4% (w/w) conjugated linoleic acid (CLA) isomers for 8 wk to determine effects of specific isomers on multiple eicosanoids in obesity. Liquid-chromatography-mass spectrometry analysis showed that compared to controls, those given *t*10,*c*12 CLA had increased liver leukotriene B_4 levels, while immunoblotting revealed that rats given either *t*10,*c*12 or *c*9,*t*11 CLA had lower liver cyclooxygenase-2. In kidney, compared to *c*9,*t*11 CLA or controls, *t*10,*c*12 CLA increased cyclooxygenase-1, 6-keto-prostaglandin $F_{2\alpha}$ and thromboxane B_2 and inhibited the in vitro production of 13-hydroxyoctadecadienoic acid and 5-, 8-, 12- and 15-hydroxyeicosatetraenoic acid. In lean compared to *fa/fa* rats, endogenous levels and in vitro production of liver and kidney 9- and 13-hydroxyoctadecadienoic acid were elevated. Previous investigations on these tissues revealed that *t*10,*c*12 CLA reduced hepatic steatosis, but increased renal damage. How these changes in eicosanoids in response to *t*10,*c*12 CLA relate to the previous findings remains to be elucidated.

ACKNOWLEDGMENTS

I would like to express my thanks to my supervisor, Dr. Harold Aukema for giving his time, assistance and experience to me. Thank you to my committee members, Dr. Carla Taylor and Dr. Elissavet Kardami for agreeing to be a part of my committee, taking the time to meet. Thank you to Dr. Carla Taylor and Dr. Peter Zahradka for providing the tissues from their research to me and giving help for my comprehension of this project. Thank you to Dr. Elissavet Kardami for giving understanding and encouragement to me.

Thank you to Joy Gauthier and Tanja Winter who helped me with learning Western blotting and LC-MS. I also would like to say thanks to all members from Dr. Harold Aukema' lab including Naser Ibrahim, and Stephnie Caligiuri and thank you to Maria Baranowski and Lisa Rigaux for animal care

Thank you to Ren Li who helped with using softwares of protein and Western blotting. Thank you to Dannielle Stringer, Vanessa Declercq and Nan Wu who gave their experiences with extraction of protein from tissues to me, and discussed Western blotting with me.

I also cannot forget to thank my daughter, my husband, my parents, my sister and my friends for their encouragement, friendship and support over the past years.

LIST OF ABBREVIATIONS

***c*9,*t*11CLA**	***cis*-9, *trans*-11 CLA**
***t*10, *c*12 CLA**	***trans*-10, *cis*-12 CLA**
AA	**arachidonic acid**
ALT	**alanine transaminase**
AST	**aspartate aminotransferase**
apoB	**apolipoprotein B**
CD	**conjugated diene**
CLA	**conjugated linoleic acid**
COX	**cyclooxygenase**
cPLA$_2$	**cytosolic phospholipases A$_2$**
CRP	**C-reactive protein**
CYP	**cytochrome P450**
DDH$_2$O	**double-distilled H$_2$O**
EETs	**epoxyeicosatrienoic acids**
ESI	**electrospray ionization**
fa/fa	**obese Zucker**
***fa/fa* 9,11 CLA**	***fa/fa* rats fed control diet plus 0.4% (w/w) *c*9,*t*11 CLA isomer**
***fa/fa* 10,12 CLA**	***fa/fa* rats fed control diet plus 0.4% (w/w) *t*10,*c*12 CLA isomer**
FLAP	**5-lipoxygenase-activating protein**
GLUT-4	**glucose transporter-4**

GFR	**glomerular filtration rate**
HETE	**hydroxyeicosatetraenoic acid**
HODE	**hydroxyoctadecadienoic acid**
HPLC	**high performance liquid chromatography**
5-S-HPETE	**5 (S)-hydroperoxy eicosatetraenoic acid**
H-PGDS	**hematopoietic PGDS**
IDV	**integrated density volume**
IκB	**inhibitor of kappa B**
Iκk	**IκB kinase**
IRS-1	**insulin receptor substrate 1**
JNK	**c-Jun N-terminal kinase**
6-keto-$PGF_{1\alpha}$	**6-keto-prostaglandin $F_{1\alpha}$**
LA	**linoleic acid**
LC-MS	**liquid chromatography-mass spectrometry**
LDL	**low-density lipoprotein**
L-FABP	**liver fatty acid binding protein**
LOX	**lipoxygenase**
L-PGDS	**lipocalin-type PGDS**
LTA_4	**leukotriene A_4**
LTB_4	**leukotriene B_4**
LTC_4	**cysteinyl leukotriene C_4**

LTD_4	**cysteinyl leukotriene D_4**
LTE_4	**cysteinyl leukotriene E_4**
MAPK	**mitogen-activated protein kinase**
MCP-1	**monocyte chemoattractant protein-1**
mPGES	**microsomal PGE synthase**
MNC	**mononuclear cell**
MS	**mass spectrometry**
NF-κB	**nuclear factor-kappa B**
NIK	**NF-κB –inducing kinase**
PG	**prostaglandin**
PGDS	**prostaglandin D_2 synthase**
PGES	**prostaglandin E synthase**
PGFS	**prostaglandin F synthase**
PGE_2	**prostaglandin E_2**
$PGF_{2\alpha}$	**prostaglandin $F_{2\alpha}$**
PGG_2	**prostaglandin G_2**
PGH_2	**prostaglandin H_2**
PGHS	**prostaglandin G/H synthase**
PGIS	**prostacyclin synthase**
PGI_2	**prostacyclin**
PKC	**protein kinase C**

PLA_2	**phospholipase A_2**
ROS	**reactive oxygen species**
$sPLA_2$	**secretory phospholipase A_2**
SCD-1	**stearoyl-CoA desaturase-1**
SDS-PAGE	**sodium dodecyl sulfate polyacrylamide gel**
SREBP-1α	**sterol responsive element-binding protein-1α**
TNF-α	**tumour necrosis factor-α**
TXB_2	**thromboxane B_2**
TXs	**thromboxanes**
TXS	**thromboxane A_2 synthase**
UCP-2	**uncoupling protein-2**
VLDL	**very low density liporotein**

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INTRODUCTION

Obesity is a component of the metabolic syndrome which is defined by the presence of three of the five following conditions: obesity, hypertension, elevated plasma triglyceride, low HDL cholesterol and increased fasting glucose (Beilby, 2004). Its prevalence is increasing in virtually all populations and age groups worldwide. Metabolic syndrome also is associated with increased risk of diabetes, atherosclerosis, fatty liver, peripheral insulin resistance and kidney lesions. Low-grade inflammation is important in the development of the metabolic syndrome and its associated complications. CLA has been postulated to exert favorable effects on obesity (Evans et al, 2002) and in the Zucker rat may improve peripheral insulin resistance (Houseknecht et al, 1998), attenuate glomerular lesions (Drury et al, 2009) and ameliorate hepatosteatosis (Gudbrandsen et al, 2009) by reducing inflammation via lipid metabolism. In vitro studies have demonstrated that CLA can alter eicosonoid formation. However, the effect of specific CLA isomers on eicosanoid metabolism in obesity in vivo remains to be elucidated. The present study aims to explore the effect of dietary CLA isomers on eicosanoid metabolism in liver and kidney in a rat model of obesity with metabolic syndrome.

1. LITERATURE REVIEW

1-A. Introduction

1-A-1. Inflammation – diseases link

1-A-1-1. Inflammation

Inflammation is a protective response to remove organisms and necrotic cells or tissue from damaged tissues. A critical component of inflammation is the accumulation of leukocytes at the site of injury. At the same time that leukocytes eliminate injurious agents and heal damaged tissue, they also can cause tissue damage by releasing enzymes, chemical mediators, and toxic oxygen radicals. Chronic inflammation is caused by persistent infection, prolonged exposure to toxic agents, and is characterized by the presence of lymphocytes and macrophages at inflammatory sites (Lawrence et al, 2002). During chronic inflammation, monocytes are attracted to sites of inflammation by chemotactic chemical mediators and adhesion molecules, and transformed into macrophages which secrete a variety of biological products that can damage tissue. These chemical mediators include eicosanoids (i.e., prostaglandins, leukotrienes) as well as cytokines [eg. TNF-α (tumor necrosis factor-α), interleukins, and interferons], chemokines [eg. MCP-1 (monocyte chemoattractant protein-1)], nitric oxide (NO), and oxygen-derived free radicals (Chung et al, 2009). These mediators contribute to vasodilation, increased vascular permeability, chemotaxis, leukocyte adhesion, stimulation of extracellular matrix, fibroblast proliferation, collagen deposition and angiogenesis (Dray, 1995).

1-A-1-2. Chronic inflammation and diseases

Chronic inflammatory processes are linked to a number of diseases including

atherosclerosis, cancer, diabetes, metabolic syndrome, and obesity. For instance, during the development of atherosclerosis, oxidized low-density lipoprotein (LDL) mediates the formation of foam cells, which are activated macrophages laden with lipid; these activated macrophages express inflammatory molecules and form fatty streak lesions (Nakamura et al, 2008). Smooth muscle cells also can be activated by inflammatory cytokines and growth factors, causing them to migrate to the subendothelial space and contributing to streak lesion formation and production of inflammatory mediators (Chung et al, 2009). Tumourigenesis also is triggered by tumour cell- and macrophage-derived chemokines. These chemokines are assumed to elicit anti-tumour effects through activation of macrophages (Rollins, 2006); however, they are also pro-angiogenic and promote proliferation and growth of tumour cells through anti-apoptotic effects (Chen et al, 2001 and Modugno et al, 2005).

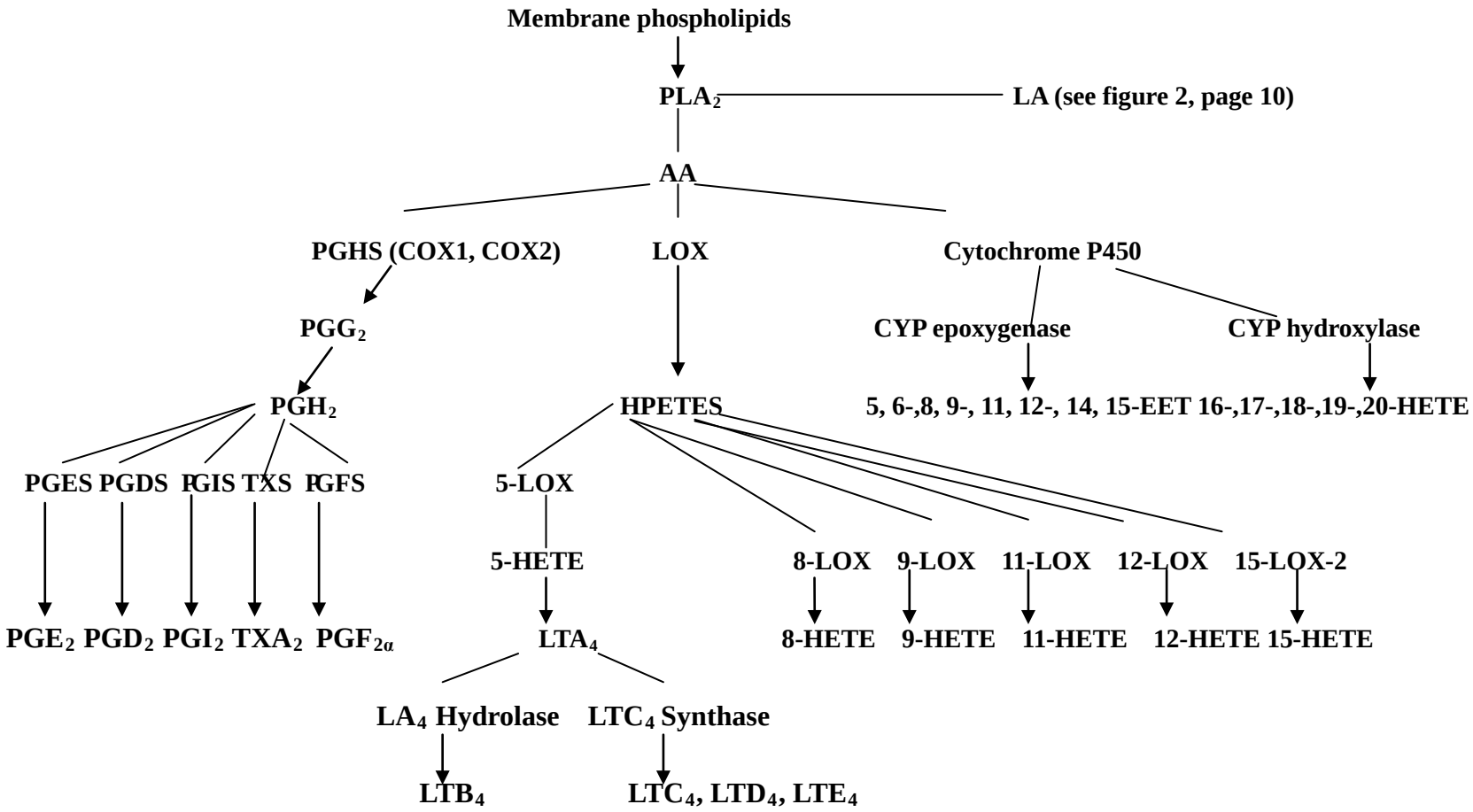
The increase in circulating inflammatory molecules is lower than that found during acute inflammation; hence these chronic diseases have a chronic low-grade inflammatory response (Chung HY et al 2009).

1-A-2. Inflammation – eicosanoids link

1-A-2-1. Metabolism of arachidonic acid (AA) and eicosanoids

Eicosanoids are generated via the metabolism of arachidonic acid (AA, C20:4) (Figure 1). AA is a polyunsaturated fatty acid in phospholipids of cell membranes. When tissues are stimulated by diverse physiologic and pathologic factors, AA is liberated from membrane phospholipids by the action of phospholipase A_2 (PLA_2). AA then can be

Figure 1. AA metabolism in COX, LOX and CYP pathways



metabolized to eicosanoids through three pathways: the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway, and the cytochrome P450 (CYP) monooxygenase pathway to form prostanoids, hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs), and epoxyeicosatrienoic acids (EETs) (Wang and Dubois, 2007).

1-A-2-1-1. Release of AA from phospholipids

The first step in AA metabolism is the release of AA from membrane phospholipids. PLA_2 is responsible for catalyzing the hydrolysis of ester bonds at the sn-2 position of phospholipids and releasing fatty acids [e.g., AA or linoleic acid (LA)]. PLA_2 consists of several isoforms including cytosolic ($cPLA_2$) and secretory phospholipases A_2 ($sPLA_2$): $cPLA_2$ is specific for AA in various cell types; $sPLA_2$ contributes to the release of AA and other unsaturated fatty acids (Stachowska et al, 2007a).

1-A-2-1-2. Cyclooxygenase (COX) pathway

AA is catalyzed via the COX pathway to form prostanoids, which include prostaglandins (PGs) and thromboxanes (TXs) (Cipollone et al, 2008). The key steps are the formation of unstable prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by COX which is also called prostaglandin G/H synthase (PGHS). PGH_2 is then converted into more stable PGs including PGE_2, PGD_2, PGF_{2a}, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) via specific PG synthases. PGHS has two isomers: COX1 and COX2. Because of the difference of tissue expression, these two isomers exert different biological effects (Wang and Dubois, 2007). COX1 is constitutively expressed at relatively stable levels in most normal tissue to mediate physiological responses to circulating hormones. It is present at higher concentrations in kidney, stomach and vascular endothelium (Cipollone et

al, 2008). In contrast, COX2 is normally present at low levels in tissues but is induced by various cytokines, growth factors and tumour promoters through the activation of NF-κB (Cipollone et al, 2008). NF-κB is one of the transcriptional factors that regulate COX2 synthesis, and there are two binding sites for NF-κB in the promoter region of the COX2 gene (Hernandez et al, 1999). COX2 is expressed in inflammatory sites, where PG synthesis is enhanced to mediate pathological processes.

PGE_2 is synthesized by prostaglandin E synthase (PGES) which has three isoforms known as microsomal PGE synthase-1 (mPGES-1), microsomal PGE synthase-2 (mPGES-2), and cytosolic PGE synthase (cPGES). Of these enzymes, mPGES-1 is induced by cytokines and inflammatory stimuli to generate PGE_2 through the COX2 pathway (Kamei et al, 2004); therefore, COX2 and mPGES-1 appear to be coregulated (Trebino et al, 2003 and Stichtenoth et al, 2001). Conversely, cPGES is constitutively expressed in a wide variety of cells (Trebino et al, 2003). On the other hand, mPGES-2 can couple with both COX2 and COX1 to produce PGE_2, but generally does not result in as marked an increase in PGE_2 as with mPGES-1 in inflammation (Mattila et al, 2009).

For the synthesis of PGD_2, two distinct types of PGD_2 synthase (PGDS), the lipocalin-type PGDS (L-PGDS) and the hematopoietic PGDS (H-PGDS) have been identified. L-PGDS is a secreted protein expressed in the central nervous system, retina, male and female genital organs of various mammals, and cortex and outer medulla of kidney. H-PGDS is a cytosolic protein detected in most of tissues (Urade and Hayaishi, 2000).

PGI_2 is generated by the action of PGI_2 synthase (PGIS). PGIS is constitutively

expressed in endothelial cells. Once generated by these cells, PGI_2 is rapidly converted to its stable metabolite, 6-keto-$PGF_{1\alpha}$. PGI_2 activities include vasodilation and inhibition of leukocyte and neutrophil adhesion to endothelial cells (Harada et al, 1999).

Thromboxane A_2 synthase (TXS) is responsible for TXA_2 generation. TXS is localized to blood platelets, monocytes and vascular smooth muscle cells and acts as a vasoconstrictor (Isozaki et al, 1994). The balance between TXA_2 and PGI_2 plays an important role in vascular homeostasis (Cipollone et al, 2008).

PGF has two isomers, namely, $PGF_{2\alpha}$ and 9α,11β-PGF_2. $PGF_{2\alpha}$ can be converted from PGE_2 and PGH_2 by PGE_2 9-ketoreductase and PGH 9,11-endoperoxide reductase, respectively (Suzuki et al, 1999). PGF synthase (PGFS) which catalyzes PGD_2 to 9α,11β-PGF_2 (PGD_2 11-ketoreductase) and PGH_2 to $PGF_{2\alpha}$ (PGH_2 9, 11-endoperoxide reductase) has two isoforms (Beasley et al, 1987): 1) The bovine lung-type enzyme highly expressed in the lung is primarily responsible for the conversion of PGF_2 from PGD_2; 2) the bovine liver-type enzyme highly expressed in the liver is mainly for the conversion of PGF_{2a} from PGH_2. PGFS does not catalyze the reduction of PGE_2. The similar biological effects of 9α,11β-PGF_2 and $PGF_{2\alpha}$ include contraction of bronchial smooth muscle and airway (Chen et al, 1992). The PGE_2-9-ketoreductase catalyzes the conversion of PGE_2 to $PGF_{2\alpha}$. This enzyme is present in kidney to regulate papillary activity through PGE_2/$PGF_{2\alpha}$ (Rathaus et al, 1986).

1-A-2-1-3. Lipoxygenase (LOX) pathway

In the LOX pathway, there are four major isoforms, namely 5-, 8-, 12- and 15- LOX. The digits designate the position where the molecular oxygen is inserted into free and/or

esterified polyunsaturated fatty acids. LOX can catalyze AA, LA, and other polyunsaturated fatty acids to form bioactive metabolites. The first step is that these four isoforms of LOXs catalyze AA to generate the corresponding hydroperoxyeicosatetraenoic acids (HPETEs), respectively. HPETEs are sequentially metabolized into the corresponding hydroxyeicosatetraenoic acid (HETEs) (Patel et al, 2008).

5-LOX is induced by pro-inflammatory stimuli or tumours. It can convert AA into 5-S-HPETE through oxygenation and subsequently an unstable epoxide leukotriene A_4 (LTA_4) through dehydration. 5-HPETE can be spontaneously reduced to 5-HETE. LTA_4 can be either hydrolyzed by LTA_4 hydrolase to give rise to leukotriene B_4 (LTB_4) or conjugated with glutathione by cysteinyl leukotrien C_4 (LTC_4) synthase to produce cysteinyl leukotrienes including LTC_4, LTD_4 and LTE_4 (Pidgeon et al, 2007). The activity of 5-LOX requires membrane-embedded 5-LOX-activating protein (FLAP) to transfer AA to 5-LOX to enhance the oxygenation of AA to 5-S-HPETE and subsequent dehydration to LTA_4 (Evan et al, 2008).

For 12-LOX, there are four types: platelet type, leukocyte type, epidermal type and a new mammalian 12-LOX underlying the generation of products with R-chirality (12(R)-HETE). 12-LOX isozymes are expressed in various types of cells, such as smooth muscle cells, keratinocytes, endothelial cells and tumour cells as well as in leukocytes and platelets (Kuhn et al, 1999). Platelet-type 12-LOX catalyzes AA to synthesize 12(S)-HETE, whereas leukocyte-type 12-LOX can also synthesize 15(S)-HETE as well as 12(S)-HETE (Pidgeon et al, 2007).

15-LOX includes 15-LOX-1 and 15-LOX-2. 15-LOX-1 is distributed in reticulocytes,

eosinophils and airway epithelial cells as well as in macrophages and in atherosclerotic lesions. Both LA and AA can be substrates of 15-LOX. 15-LOX-1 preferentially converts LA to 13-S-hydroxyoctadecadienoic acid (13-S-HODE), but metabolizes AA to 15-(S)-HETE poorly. 15-LOX-2, on the other hand, preferentially converts AA to 15-(S)-HETE, but metabolizes LA poorly (Pidgeon et al, 2007). With the modification of nomenclature for LOXs, all leukocyte-type 12-LOXs and the reticulocyte-type 15-LOXs are grouped together because of the similarity of their enzymatic properties and amino acid sequence (Kuhn et al, 1999).

8-LOX converts AA to 8-HETE and it is expressed in mouse skin and brain. 9-LOX converts AA to 9-HETE. Little is known about the function of 8-HETE and 9-HETE in normal and diseased tissues. However, recent findings have shown that 8-HETE and 9-HETE promote tumor growth (Shureiqi and Lippman, 2001) and have chemotactic effects (Potter et al, 1985).

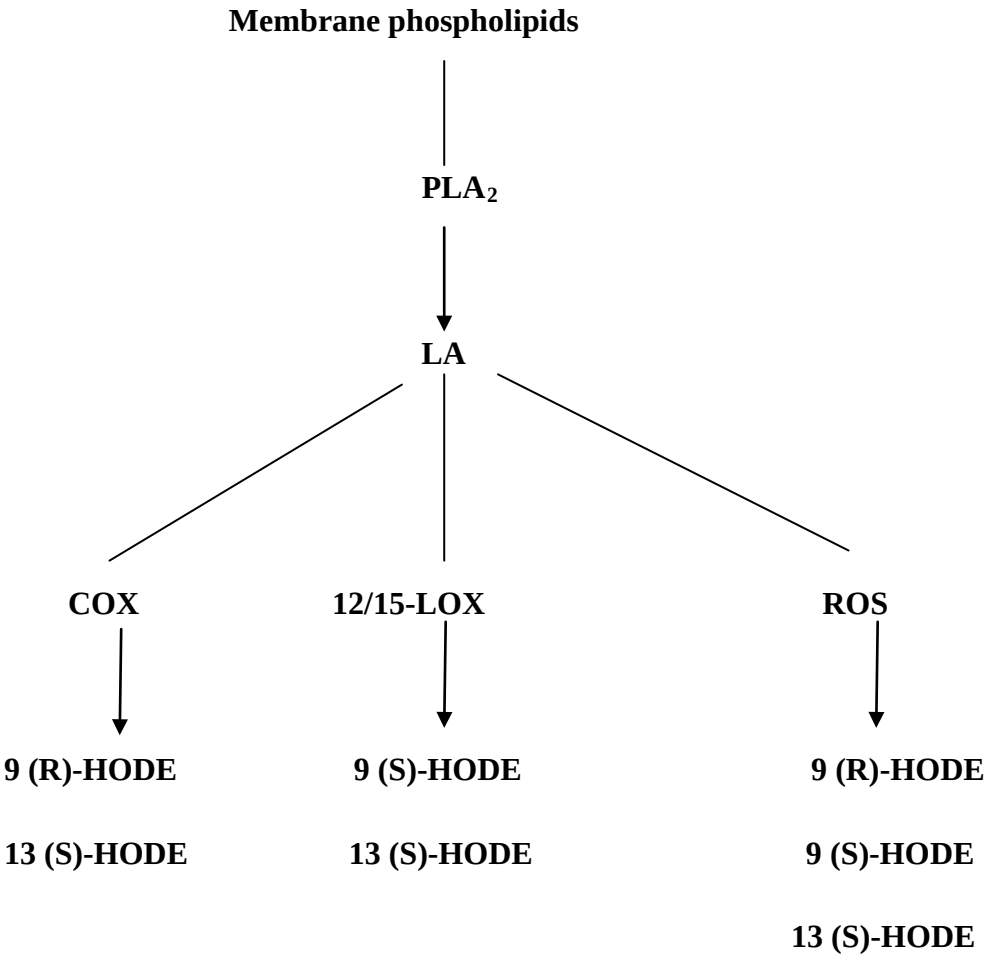
1-A-2-1-4. Cytochrome P450 (CYP) pathway

The CYP450 pathway includes CYP epoxygenases and CYP hydroxylases. The CYP epoxygenases families including CYP1A, CYP2B, CYP2C and CYP2J convert AA to 5,6-, 8,9-, 11,12-, and 14,15-EET. CYP epoxygenases were first isolated from the human heart and liver, and also are expressed in the kidney (Gross et al, 2005). CYP hydroxylase families including CYP4A, CYP3A and CYP4F catalyze the formation of HETEs including 16-, 17-, 18-, 19-, and 20-HETE (Wang and Dubois, 2007).

1-A-2-2. Generation of hydroxylinoleic acids (HODEs) from linoleic acid (LA)

13-HODE and 9-HODE are two isomers of oxidation metabolites of LA (Figure 2).

Figure. 2 HODE formation from LA



The synthesis of 9(R)-HODE is mediated by the COX pathway (Engels et al, 1986) and 9(S)-HODE is formed by 12/15-LOX (Hatley et al, 2003). 13(S)-HODE is converted by both 12/15-LOX and COX (Lee et al, 2005). Both 13-HODE and 9-HODE also can be formed by non-enzymatic actions (Lee et al, 2005).

The role of 13- HODE in vascular system is controversial. It has been believed for long time that over-expression of 15-LOX and excessive 13-HODE and 9-HODE in macrophages are pro-atherogenic because 13-HODE up-regulates the CD36 scavenger receptor expression, which then increases the uptake of oxidized LDL by macrophages (Wittwer and Hersberger, 2007). However, 13-HODE also up-regulates the expression of ATP binding cassette transporter A1 (ABCA1), which increases cholesterol efflux from macrophages (Wittwer and Hersberger, 2007 and Hersberger and Eckardstein, 2003). This finding is consistent with another study in an atherosclerotic rabbit model (Shen et al, 1996) which showed that excessive production of 13-HODE resulting from over-expression of 15-LOX in macrophages reduced the onset of streak lesions. Others (Fang et al, 1999) have confirmed that 13- HODE originating from endothelium reduces the adhesion of platelets to the endothelial surface, increases PGI_2 production in the fetal bovine aortic endothelial cells, decreases thromboxane production in platelets, and thus plays a positive role in the vascular system. In contrast, this group had previously reported that 13-HODE and 9-HODE decrease the production of PGI_2 from human umbilical vein endothelial cells (Kaduce et al, 1989).

1-A-2-3. Physiological effects of eicosanoids

1-A-2-3-1. Physiological effects of eicosanoids on liver

There are three types of cells in liver including heptaocytes, which is the main liver cell type, Kupffer cells, which form the liver macrophages, and extrahepatic endothelial cells. A direct comparison of the capacity of three types of cells to produce eicosanoids (Kuiper et al, 1988) showed that Kupffer cells and endothelial cells produced much higher quantity of eicosanoids. COX1 is constitutively expressed in liver; but, it was found to be increased in cirrhotic rat livers (Gracia-Sancho et al, 2007). COX2 is inducible in inflammatory conditions (Bhave et al, 2008). PGE_2 (Dieter et al, 2002) is an important regulator of liver functions. PGE_2 regulates the sinusoidal flow through regulation of contraction of hepatic stellate cells and has an anti-fibrosis effect by inhibiting proliferation of stellate cells. Furthermore, PGE_2 generated from Kupffer cells downregulates TNF-α and PGE_2 generated from hepatocytes and induces glycogenolysis. PGD_2 (Kuiper et al, 1988) is another product mainly generated from non-parenchymal cells. Similar to PGE_2, PGD_2 participates in the control of the sinusoidal liver blood flow. Moreover, PGD_2 might inhibit platelet aggregation and enhance the glucose output by parenchymal cells through cellular communication between the various liver cell types. $PGF_{2\alpha}$ along with PGE_2 modifies hepatic functions including biliary flow and glucose output through coordination of intercellular Ca^{2+} signals (Koukoui et al, 2006). Endothelial cells in liver form minor amounts of PGI_2. PGI_2 can maintain hepatic tissue blood flow and prevent liver from ischemia/reperfusion injury through vasodilatation and inhibition of leukocytes activation (Harada et al, 1999). The study of Isozak et al (Isozaki et al, 1994) showed that a TXA_2

synthetase inhibitor but not a COX inhibitor attenuated postischemic injury of the liver through suppression of TXB_2 and increased 6-k-$PGF_{1\alpha}$/TXB_2 confirming that TXB_2 counteracts the effect of PGI_2.

LTs generated from 5-LOX are involved in inflammation through enhancement of leukocyte migration and degranulation of neutrophils (Urade et al, 1996). The physiological role of 15-LOX is involved in programmed degradation and turnover of peroxisomes in hepatocytes (Yokota et al, 2001). Mathews et al (1994) further confirmed that the increased metabolites of lipid peroxidation including 12-, 11- 8- and 9-HETE were involved in liver injury.

The role of CYP450 in liver is that CYP450s including 1A2, 2A6, 2C8, 2C9, 2C19, 2D6 and 3A4 are implicated in drug metabolism. Of these enzymes, CYP3A is the target of most clinical drugs in humans (Morgan, 1997). CYP 4A and CYP 2E are induced in rat liver to mediate lipid peroxidation and result in oxidative stress and liver injury (Robertson et al, 2001). The enzymes involved in eicosonoid metabolism and eicosanoids in liver are summarized in Table 1 and Table 2.

1-A-2-3-2. Physiological effects of eicosanoids on kidney

Eicosanoids maintain renal function, body fluid homeostasis, and blood pressure through vasodilation or vasoconstriction in an autocrine or paracrine fashion. TXA_2, PGE_2 and PGI_2 are the primary eicosanoids of the COX pathway that regulate renal blood flow and glomerular filtration (GFR) (Imig, 2000). PGE_2 counteracts renal vasoconstriction induced by angiotensin to increase renal blood flow. PGI_2 dilates the glomerular vasculature to counteract the pro-platelet aggregation and vasoconstriction actions of

Table 1. Enzymes involved in eicosanoid metabolism in liver and kidney

Tissues	PGS	LOX	CYP
Kidney	COX1, COX2, PGES, m-PGES-1, m-PGES-2, cPGES PGIS, TXBS, L-PGDS, PGFS (Komers et al, 2005; Imig, 2000, Harris, 2000 and Hao and Breyer, 2008)	5-LOX, 12/15-LOX, 15-LOX-2, 8-LOX, 9-LOX (Gonzalez-Nunez et al, 2005; Imig, 2006 and Jim et al, 1982)	CYP2C23,CYP2C11, CYP4A,CYP4F, CYP2J2 (Yu et al, 2000; Sang et al, 2003; Imig, 2006; Dey et al, 2004; Kroetz and Xu, 2005)
Liver	COX1, COX2, PGES, m-PGES-1, m-PGES-2, cPGES PGIS, TXBS, L-PGDS, PGFS (Isozaki et al, 1994; Harada et al, 1999; Dieter et al, 2002; Kuiper et al, 1988; Gracia-Sancho et al, 2007 and Bhave et al, 2008）	5-LOX, 12/15-LOX, 15-LOX-2, 8-LOX, 9-LOX, LTC_4 synthase (Yokota et al, 2001; Urade et al, 1996 and Zhu et al, 2008)	CYP 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 3A4 (Morgan, 1997 and Robertson et al, 2001)

Table 2. Eicosanoids in liver and kidney

Tissues	Prostanoids	HETEs	EETs
Kidney	PGE_2, 6-keto-$PGF_{1\alpha}$, TXB_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (Hao and Breyer, 2008)	5-HETE, 12-HETE, 15-HETE, LTB_4, 8-HETE, 9-HETE (Jim et al, 1982)	5,6-,8,9-,11,12-,14,15-EETs 19- and 20-HETE, CYP 4A CYP 2E (Wang et al, 2003)
Liver	PGE_2, 6-keto-$PGF_{1\alpha}$, TXB_2, PGD_2, $PGF_{2\alpha,}$ PGI_2 (Isozaki et al, 1994; Harada et al, 1999; Dieter et al, 2002; Kuiper et al, 1988 ; Gracia-Sancho et al, 2007; Bhave et al, 2008 and Koukoui et al, 2006)	5-HETE, 12-HETE, 15-HETE, 8-HETE, 9-HETE, LTB_4 LTC_4, LTC_4 , LTE_4 (Yokota et al, 2001; Urade et al, 1996; Zhu et al, 2008)	5,6-,8,9-,11,12-,and14, 15-EETs, 19- and 20-HETE, CYP 4A CYP 2E (Wang and Dubois, 2007 and Gross et al, 2005)

TXA_2. L-PGDS is viewed as a biomarker for renal injury associated with diabetic complications. However, the function of H-PGDS remains unclear (Hao and Breyer, 2008). Traditionally, COX1 is viewed as a constitutive housekeeping enzyme, responsible for maintaining basic physiological functions, including cytoprotection of the gastric mucosa and control of platelet aggregation. In kidney, COX1 localized to collecting ducts is hypothesized to contribute to natriuresis (Komers et al, 2005). Conversely, COX2 is induced in inflammation. However, recent studies have shown that COX2 is also constitutively expressed in macula densa cells to regulate chloride reabsorption and renin production and release (Harris, 2000).

AA metabolites in the LOX pathway regulate renal haemodynamics as well as inflammatory actions. For example, a study in rat nephritis showed that 5-LOX associates with the increased glomerular cell proliferation (Wu and Lianos, 1993). It also was found that the increased mRNA and protein levels of 12-LOX in rat mesangial cells correlate with histological damage in diabetic nephropathy (Kang et al, 2001). This increased production of 12-LOX-generated 12-HETE in mesangial cells promotes cell growth and matrix protein expression through mitogen-activated protein kinase (MAPK) and angiotensin II (Reddy et al, 2002). 12/15-LOX and 12-HETE can constrict renal vessels and glomerular mesangial cells, decreasing renal blood flow and GFR. This effect appears to be through increased expression of angiotensin II type 1 receptor (Gonzalez-Nunez et al, 2005). 12-HETE can upregulate the angiotensin II receptor through activation of p38 MAPK (Xu et al, 2008). The angiotensin II can induce mesangial cell growth and extracellular matrix protein (fibronectin) production (Xu et al, 2005). LTs increase

glomerular and capillary permeability and are associated with proteinuria and interstitial nephritis (Imig, 2006). 8-HETE and 9-HETE are formed by glomerular mesangial cells, their functions are unclear (Jim et al, 1982).

With respect to the role of CYP enzymes in kidney, in the renal vasculature, 20-HETE constricts renal vessels, but EETs dilate renal arterioles. In renal tubular segments, 20-HETE inhibits sodium reabsorbtion through inhibition of Na^+-K^+-ATPase activity in the proximal tubule, and EETs also inhibit sodium reabsorbtion in the collecting duct (Wang et al, 2003). The enzymes involved in eicosonoid metabolism and eicosanoids in kidney are summarized in Table 1 and Table 2.

1-A-2-4. Regulation of eicosanoids by inflammation

1-A-2-4-1. Up-regulation of eicosanoids in the COX and LOX pathways by inflammation

Various inflammatory cytokines generated in inflammation are capable of activating synthesis of eicosanoids. For example, TNF-α and IL-1β promote the release of fatty acids from membrane phospholipids. In the HaCaT skin keratinocyte cell line, Anthonsen and his group (Anthonsen et al, 2001) reported that TNF-α and IL-1β induced release of AA through activation of the mitogen activated protein kinase kinase kinase (MAP3K) family including NF-κB-inducing kinase (NIK). This sequentially induces the phosphorylation of $sPLA_2$ and $cPLA_2$ to release fatty acids and activation of NF-κB.

Specific cytokines also can enhance the expression of the enzymes responsible for the synthesis of individual eicosanoids. COX2 can be induced by cytokines and growth factors

through the activation of NF-κB. For example, in 1321N1 astrocytoma cells (Hernandez et al, 1999), it was demonstrated that TNF-α invokes the expression of COX2 through activation of NF-κB via two NF-κB-binding sites. TNF-α also activates $cPLA_2$ in these cells. Cowburn et al reported (Cowburn et al, 1999) that in human blood eosinophils, IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) enhance the gene transcription and protein synthesis of 5-LOX and FLAP as well as activating $cPLA_2$. Similarly, Spanbroek et al reported that IL-4 up-regulates 5-LOX and FLAP genes in cultured dendritic cells (Spanbroek et al, 2001). 5-LOX products in turn enhanced nuclear translocation of p50 and p65 subunits to activate NF-κB (Horrillo et al, 2010). In porcine vascular smooth muscle cells, IL-1, IL-4, and IL-8 induce mRNA and protein levels of 12-LOX (Natarajan et al, 1997). 12-HETE also activates protein kinase C (PKC), p38 MAPK and c-Jun N-terminal kinase (JNK) and enhances inflammatory cytokines such as MCP-1, TNF-α and IL-6 in rat kidney (Reddy et al, 2002) and mouse liver (Martinez-Clemente, et al, 2010). Several lines of study showed that IL-4 (Conrad et al, 1992) and IL-13 (Nassar et al, 1994) enhance 15-LOX mRNA and protein levels in cultured monocytes.

There also is much other evidence that the elevated levels of eicosanoids are associated with different inflammatory diseases. For instance, it was found that PGE_2 released from rectal mucosa is elevated in patients with ulcerative colitis (Rampton et al, 1980). PGE_2, LTB_4 and TXB_2 were found to be elevated in the serum of alcoholic liver injury and associate with the severity of liver injury (Nanji et al, 1993). $PGF_{2\alpha}$, PGE_2, TXB_2 (Trang et al, 1977) and PGI_2 levels (Brodie et al, 1980) in knee joint fluid and

$PGF_{2\alpha}$ metabolites in both plasma and urine were increased (Goetzl et al, 1982) in rheumatoid arthritis. PGD_2 level in bronchoalveolar lavage, peripheral blood and urine was found to be increased (Holgate et al, 1991) and 2, 3 dinor-9α-11 β -PGF_2 level in urine was elevated (Higashi et al, 2010) in asthma. PGE_2, $PGF_{2\alpha}$, PGD_2 and TXB_2 also play a pivotal role in atherosclerosis (Fresno M et al, 2008) .

In addition to involvement in liver injury after partial hepatectomy (Urade et al, 1996) and glomerular cell proliferation in nephrotoxic serum nephritis in rat (Wu and Lianos, 1993), 5-LOX and its metabolites participate the eosinophil chemotactic responses as chemotactic factors, and are implicated in rheumatoid arthritis, asthma (Goetzl et al, 1982) and human arterial wall atherogenesis (Spanbroek et al, 2003). As pointed out above, over expression and production of 12/15-LOX and 12-HETE contribute to the expression of ANG II and kidney damage in diabetic nephropathy (Kang et al, 2001). It has long been known that 12/15-LOX is pro-atherogenic via promotion of macrophage adhesion to endothelial cells (Funk, 2006) and the production of oxidized metabolites of 12/15-LOX including 13-HODE and 9-HODE. These latter products are pro-atherogenic because they increase the uptake of oxidized LDL in macrophages (Wittwer et al, 2007). Additionally, it was found that the expression of 12/15-LOX in adipose tissues contributes to the secretion of inflammatory cytokines in high fat diet-induced insulin resistance of mice (Chakrabarti et al, 2009).

1-A-2-4-2. Down-regulation of eicosanoids in the CYP pathway by inflammation

As opposed to the general up-regulation of eicosanoids derived from COX and LOX pathways, inflammation and infection have long been known to down-regulate the

majority of activities and expressions of CYP enzymes (e.g.CYP1A2, 2A1, 2C6, 2C7, 2C11, 2C23, 2E1, 3A2, and 4F4 in the rat) in liver and extraheptic tissues (Morgan et al, 2008). Since CYP enzymes also produce reactive oxygen species (ROS), and EETs have anti-inflammatory as well as vasodilatory properties, this down-regulation aids in reducing oxidative stress, allows the execution of the inflammatory response and/or combats the hypotension which occurs in inflammation (Aitken et al, 2006). However, this state of decreased CYP enzymes also can exert deleterious effects: 1) it can reduce drug clearance and therapeutic effect of drugs which are needed to be converted to their pharmacologically active metabolites by CYPs in the liver (Morgan, 1997); 2) in the kidney, it can contribute to the formation of hypertension since EETs can serve as a renal vasodilator and inhibit the tubular resorption of tubular sodium (Wang et al, 2003).

Oxidative stress and pro-inflammatory cytokines are involved in the mechanisms by which CYP enzymes are down- regulated (Aitken et al, 2006). Pregnane X receptor (PXR) is one of the transcription factors underlying the synthesis of CYP enzymes. Retinoic acid receptor (RXRα) is the dimerization partner of PXR. After the activation of NF-κB by cytokines or oxidative stress, NF-κB p65 can interact with the PXR/RXRα complex to prevent its binding to its consensus DNA sequences, thus inhibiting the transactivation by the PXR/RXRα complex (Gu et al, 2006). In addition to NF-κB, C/EBPα is another important transcription factor which regulates expression of CYP enzymes (Aitken et al, 2006). In inflammation, the expression of C/EBPα is decreased and down-regulates the synthesis of CYP enzymes (Morgan, 1997). In contrast, CYP4A and CYP2E1 are induced in inflammation (Aitken et al, 2006). The mechanism is that the induction of CYP4A is

mediated by PPAR-α. The activated PPAR-α binds with RXR as a heterodimer, and then binds to response elements in target genes (Johnson et al, 2002). For the induction of CYP2E1, the regulation by hormone is complicated (Robertson et al, 2001).

1-B. Obesity

1-B-1. Obesity and metabolic syndrome

Metabolic syndrome is known as Syndrome X with several abnormalities including obesity, hyperglycemia, hypertension, and dyslipidemia. According to the definition of metabolic syndrome from the National Cholesterol Education Program's Adult Treatment Panel III report (ATP III), the diagnostic criteria includes three of the five following abnormalities: 1) obesity : waist circumference > 102 cm for men and > 88 cm for women; 2) raised triglycerides : triglycerides ≥ 1.7 mmol/L; 3) reduced high-density lipoprotein (HDL): HDL < 1.04 mmol/L for men and < 1.30 mmol/L for women; 4) elevated blood pressure: ≥ 130/ ≥ 85 mm Hg; 5) increased plasma glucose: fasting glucose ≥ 6.1 mmol/L (Beilby, 2004). Currently, there is an increasing trend in obesity in all populations and age groups worldwide, and previous research has demonstrated that individuals with metabolic syndrome are at high risk for developing diabetes mellitus, hepatic steatosis and kidney disease as well as increased mortality from cardiovascular disease, heart attack, and stroke (Ford et al, 2002).

1-B-2. Obesity and inflammation

Several lines of evidence have confirmed that obesity is a pro-inflammatory state. For instance, the detection of lipid peroxidation, oxidative and antioxidant enzyme activities in obese fatty Zucker rats showed obesity predisposes the myocardium to oxidative stress

(Vincent et al, 1999). In obese females, Ghanim et al (2004) observed a parallel increase in NF-κB binding activity characterized by higher transcriptionally active NF-κB (p65/p50), a concomitant decrease of its inhibitors, inhibitor of kappa B (IκB), such as IκB-α or IkB-β in peripheral blood mononuclear cells (MNC), and an increased expression of genes modulated by NF-κB, including TNF-α, IL-6, migration inhibitor factor, and matrix metalloproteinase-9. Additionally, in adipose tissues, Bruun et al (Bruun et al, 2004) further reported that adipose tissue from obese compared to lean humans released more IL-8 levels in plasma. Weisberg and his group (Weisberg et al, 2003) found a prominent expression of macrophages in perigonadal adipose tissue of obese mice.

1-B-2-1. Hepatic steatosis in obesity

1-B-2-1-1. Hepatic steatosis in obesity and inflammation

Hepatic steatosis is described as an excess accumulation of fat triglycerides in hepatic parenchymal cells (hepatocytes) (Fabbrini et al, 2010). The sources of increased triglycerides accumulation in hepatic steatosis include: 1) enhanced expression of TNF-α in adipose tissue, which has been demonstrated to stimulate lipolysis, increasing fatty acid influx into the liver (Cawthorn and Sethi, 2008); 2) hyperinsulinemia, which up-regulates the expression of PPARγ and sterol responsive element-binding protein-1α (SREBP-1α) in liver, increasing de novo TG synthesis in the liver (Clément et al, 2002); 3) increased malonyl-CoA which is the first intermediate in de novo lipogenesis. This metabolite inhibits carnitine palmitoyl transferase 1 activity (CPT-1), reducing the β-oxidation of fatty acids (Fabbrini et al, 2010).

Inflammation is implicated in fatty liver. In fatty liver, oxidation of fatty acids is an

important source of ROS that leads to oxidative stress (Browning and Horton, 2004). Oxidative stress activates the inflammatory pathway, and results in the release of proinflammatory cytokines and the destruction of membranes via lipid peroxidation (Robertson et al, 2001). During lipid peroxidation, by-products of polyunsaturated fatty acid peroxidation, such as trans-4-hydroxy-2-nonenal and malondialdehyde are formed. These products themselves are potent chemoattractants for neutrophils. Ultimately, the influx of inflammatory cells into the liver can activate stellate cells, leading to collagen deposition and fibrosis (Browning and Horton, 2004).

1-B-2-1-2. Eicosanoids and hepatic steatosis in obesity

Several eicosanoids and relevant enzymes are implicated in the progression of obesity-related fatty liver disease. A study (Cao et al, 2008) of high fat-induced obesity in Sprague-Dawley male rats found that COX2 protein levels were increased in liver tissue along with increased IL-1 and TXB_2 and decreased PGI_2. COX2 was associated with liver injury as shown by histological evidence and levels of serum alanine transaminase (ALT) and aspartate aminotransferase (AST).

López-Parra (López-Parra et al, 2008) reported that 5-LOX activity and its products including LTB_4, LTD_4, and 5-HETE contributed to the damage of liver steatosis in obese mice. The administration of 5-LOX inhibitor down-regulated the free fatty acids uptake (i.e., liver fatty acid-binding protein and FAT/CD36), microsomal transfer protein and apolipoprotein B (apoB) containing VLDL-TG. Martínez-Clemente et al (Martínez-Clemente et al, 2010) demonstrated that 12/15-LOX and 12-HETE compared to 5-LOX more potently contributed to hepatic steatosis and inflammation in an experimental

mouse model of non-alcoholic fatty liver disease. Puri et al (2009) found that there was a stepwise increase in 5-HETE, 8-HETE, 11-HETE and 15-HETE during the progression from normal to nonalcoholic steatosis and steatohepatitis in human plasma samples.

As discussed above, the majority of activities and expressions of CYP enzymes are decreased in liver inflammation, which results in a reduction in drug clearance and an increased risk of a compromised therapeutic effect of drugs which are needed to be converted to their pharmacologically active metabolites by CYPs in the liver (Aitken et al, 2006). Some CYP enzymes also were found to be reduced in livers of patients with steatosis, in vivo animal models of steatosis and in vitro models of fat-overloaded cells (Lechon et al, 2009). In contrast, CYP 4A and CYP 2E are induced in the oxidative stress-mediated liver injury in fatty liver because of the regulation by PPAR-α and hormones (Robertson et al, 2001 and Johnson et al, 2002).

1-B-2-2. Kidney in obesity

1-B-2-2-1. Kidney in obesity and inflammation

Kidney inflammation in obesity contributes to the development of glomerulosclerosis and focal and segmental glomerular sclerosis. Lipoprotein deposition is a linkage between inflammation and cell injury and sclerosis in this process. Results from a study of cultured mesangial cells (Schlöndorff, 1993) have demonstrated that under normal conditions, mesangial cells can take up both LDL and oxidized LDL through LDL receptors and specific scavenger receptors for glycated or oxidized LDL as well as monocyte-macrophages which give rise to foam cells after uptake of oxidized LDL. However, the expression of LDL receptors on mesangial cells is down-regulated by

preloading cells with LDL. Therefore, in hyperlipidemia, LDL and oxidized LDL are trapped in the extracellular matrix where LDL can be oxidized. This oxidized LDL is cytotoxic to mesangial, epithelial, and endothelial cells, thereby initiating cell damage and sclerosis. Consistent with this, a study in human mesangial cells (Rovin and Tan, 1993) found that because glomerular mesangial cells possess both LDL and scavenger receptors, LDL and oxidized LDL can directly cause mesangial cells to produce mesangial matrix including components such as mesangial collagen, proteoglycans and mesangial fibronectin. LDL induces mesangial production of MCP-1 which recruits monocytes from blood and then, promotes monocytes and mesangial cells to release other factors, [e.g. platelet-derived growth factor (PDGF) and transforming growth factor-13 (TGF-13)]. LDL also can indirectly increase matrix synthesis through these events. Similar to the findings in mesangial cells, glomerular epithelial cells also express lipoprotein including VLDL and LDL receptors to mediate progressive glomerular damage (Quaschning et al, 1997).

1-B-2-2-2. Eicosanoids and kidney in obesity

The role of COX1 and COX2 in obesity-induced kidney damage is controversial. For instance, a study in 12-week-old male obese Zucker (*fa/fa*) rats (Komers et al, 2005) reported that the protein levels of renal cortex COX2 were increased along with the increased TXB_2 excretion in urine. Interestingly, COX1 protein levels which contributes to the regulation of natriuresis were decreased. Drury et al (Drury et al, 2009) found that renal COX1 protein levels did not differ between 14 wk old male of lean and *fa/fa* rats, while COX2 protein levels were increased in *fa/fa* rats.

It has long been believed that 5-LOX metabolites (Hao and Breyer, 2007) along with

ROS (Bonizzi et al, 1999) generated from 5-LOX pathway mediate inflammation and glomerular immune injury. These products are potent chemoattractants and as mentioned above, 5-LOX levels are associated with glomerular cell proliferation (Wu and Lianos, 1993). 12/15-LOX and its metabolite 12-HETE can be induced by high glucose and, it, along with angiotensin II type 1 receptor, are implicated in prediabetic kidney damage in obese *fa/fa* rats. This is thought to be mediated via upregulation of angiotensin II type 1 receptor mRNA and protein levels and MAPK activities in rat kidney (Xu et al, 2005). Both 12-HETE and ANG II induce mesangial cell growth and extracellular matrix protein (fibronectin) production (Kang et al, 2001 and Xu et al, 2008).

As indicated above, in renal vasculature, CYP4A and its metabolite 20-HETE and CYP2C and its EET metabolites have opposite vascular actions (Dey et al, 2004); however, in renal tubular segments, both 20-HETE and EETs inhibit sodium transport (Kroetz and Xu, 2005). In diet-induced obese rats, both CYP4A and CYP2C were down-regulated in renal tubules to contribute to the onset of hypertension (Wang et al, 2003). Nevertheless, in renal microvessels of Zucker rats, CYP4A protein levels were increased and CYP2C protein levels were decreased in kidney of Zucker rats (Dey et al, 2004).

1-C. Conjugated linoleic acid (CLA)

1-C-1. Sources of CLA

CLA refers to a set of positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12, octadecadienoic acid). CLA has conjugated double bonds at positions of carbons 7, 9; 8, 10; 9, 11; 10, 12; or 11, 13 which are 3-dimensional geometric combinations of *cis* and/or *trans* configurations, therefore, they are refered to as conjugated (Bassaganya-Riera et al,

2002). *Cis-9,trans*-11 CLA (*c*9,*t*11 CLA) can be formed by ruminal bacterium (*Butyrivibrio fibrisolvens*) which isomerize linoleic acid into CLA or it can be formed via conversion of vaccenic acid (*trans*-11 C18:1) by Δ9 desaturase, an enzyme present in the mammary gland and adipose tissue. Δ9 desaturase is expressed in both non-ruminant animals and ruminant animals, except that in non-ruminant species, there is a lack of the rumen-originated precursor of *c*9,*t*11 CLA (i.e. vaccenic acid). Hence, ruminant products are the main source of CLA with the *c*9,*t*11 CLA isomer being the predominant one in these products (Bassaganya-Riera et al, 2002).

CLA is found in large amounts in phospholipids and triacylglycerols of milk and dairy products, such as cheeses and yogurts and in ruminant meat, such as beef and lamb. The concentration of CLA ranges from 3.6~8.0 mg/g lipid in cheese, 3.4~6.4 mg/g lipid in milk and 2.7~5.6 mg/g lipid in ruminant meat products, depending on the species, tissues, diet and season. There are only trace amounts of CLA in vegetable oil and the fat of non-ruminant animals, ranging from 0.6~0.9 mg/g total fat (Baddini et al, 2009). Ruminant meats and milk contain 80% *c*9,*t*11 CLA and 10% *trans*-10,*cis*-12 CLA (*t*10,*c*12 CLA). Both *c*9,*t*11 CLA and t10,c12 CLA can be synthesized chemically. In commercially available crude mixtures, *c*9,*t*11CLA and *t*10,*c*12 CLA are present in approximately equal amounts. It is estimated that daily intake of CLA is 212 mg in men and 151 mg in women (Evan et al, 2002).

1-C-2. Effects of CLA isomers

Since the first observation of an inhibitory effect of CLA mixtures isolated from ground beef on skin neoplasia in mice conducted by Michael Pariza's group (Ha et al, 1987), a

flurry of research of CLA has confirmed that numerous physiologic properties are attributed to CLA, such as anti-inflammation, as well as anti-obesity, anti-atherosclerosis, anti-diabetes, and anti-carcinogenesis. These effects of CLA are isomer-specific.

1-C-2-1. Anti-obesity

Even though reports on the effects of CLA on body weight are controversial, some data from animal and human studies suggest that a mixture of CLA can reduce excess body weight gain and body fat. With respect to individual isomers of CLA, *t*10,*c*12 CLA has a more potent anti-obesity effect than *c*9,*t*11 CLA (Feitoza et al, 2009). Some potential mechanisms contribute to the anti-obesity effect： 1) Inhibition of stearoyl-CoA desaturase –1 (SCD-1) (Δ9 desaturase) which is responsible for the desaturation of saturated fatty acid to produce monounsaturated fatty acid which serves as substrate for triglycerides (Evan et al, 2002). 2) Inhibition of the differentiation of preadipocytes (Evan et al, 2002). 3) Inhibition of PPAR-γ mainly expressed in adipose tissue. PPAR-γ is the transcription factor encoding the synthesis of enzymes for lipogenesis such as fatty acid synthase. 4) Activation of PPAR-α to increase both peroxisomal oxidation (Evan et al, 2002) and mitochondrial oxidation (Martin et al, 2000) of fatty acid in liver and adipose tissue.

1-C-2-2. Anti-diabetes

The anti-diabetic property of CLA is species- and CLA isomer-specific. Studies have demonstrated that a CLA mixture improves insulin resistance in rats through activation of PPAR-γ (Houseknecht et al, 1998), and that CLA feeding is accompanied by increases in the levels of adiponectin and leptin upon activation of PPAR-γ (Zhou et al, 2008). It is proposed that the increased uptake of fatty acid by adipose tissue resulting from

PPAR-γ activation leads to a decrease in the lipid content of muscle and improvement of muscle insulin sensitivity. The anti-diabetic effect has been attributed to *t*10,*c*12 CLA (Henriksen et al, 2003).

Dietary supplementation of CLA results in the development of lipoatrophic diabetes in mice due to increased apoptosis of adipocytes and the resulting increase in TNF-α and inhibition of PPAR-γ (Tsuboyama et al, 2000). However, the prolonged treatment of genetically obese mice with *t*10,*c*12 CLA also can improve glucose tolerance via activation of PPAR-γ (Wargent et al, 2005). In addition, *c*9,*t*11-CLA can reduce inflammation in *ob/ob* mice via down-regulation of TNF–α, NF-κB DNA binding and transcriptional activity, and improve insulin resistance by regulation of insulin receptor substrate-1 (IRS-1) and glucose transporter 4 (GLUT4) expression (Moloney et al, 2007).

1-C-2-3. Anti-atherosclerosis

Many studies have shown that CLA can result in less atherosclerosis through the reduction of plasma triacylglycerols and LDL-cholesterol which are related to the reduced very-low-density lipoprotein (VLDL) and LDL observed in CLA feeding. As discussed above, *t*10,*c*12 CLA decreases monounsaturated fatty acid via the inhibition of SCD-1, resulting in reduced de novo fatty acid and TG synthesis (Evan et al, 2002). ApoB is essential for the formation and secretion of VLDL in liver cells, and acts as a ligand for the LDL receptor. *t*10,*c*12 CLA isomer inhibits the synthesis and secretion of apoB through the inhibition of triglyceride synthesis (Yotsumoto et al, 1999). Additionally, it was found that *c*9,*t*11 and *t*10,*c*12 CLA were equally effective in decreasing the synthesis of cholesterol and cholesteryl esters and reducing VLDL-cholesterol in Hep G2 cells (Yotsumoto et al,

1999).

As mentioned above, vascular smooth muscle cells, endothelial cells and macrophages play an important role in atherosclerosis (Chung et al, 2009). CLA can reduce atherosclerosis development through reduction of eicosanoids released from these cells. For instance, 15-LOX and its oxidized metabolites, 13-HODE and 9-HODE are increased to promote the progression of atherosclerosis (Funk, 2006 and Wittwer et al, 2007). CLA exerts anti-atherosclerosis through inhibiting 15-LOX activity and reducing levels of HODEs. As discussed below, in human macrophages, both CLA isomers inhibit the activity of 15-LOX-1 and production of HODEs as an inhibitor (Stachowska et al, 2007b). In pulmonary cells, *c*9,*t*11 CLA competes for substrate with LA for 13-HODE (Cho et al, 2005) to affect 15-LOX-1 activity. Another study in human coronary artery smooth muscle cells stimulated by TNF-α demonstrated that eicosanoids are implicated in the activation of smooth muscle cells. This study reported that by activating PPAR-γ *c*9,*t*11 and *t*10,*c*12 CLA down-regulate the mRNA levels of $cPLA_2$, COX-2, mPGES and the production of the PGE_2 and PGI_2 generated from vascular smooth muscle cells (Ringseis et al, 2006). Similarly, *c*9,*t*11 and *t*10,*c*12 CLA reduce the release of PGE_2, PGI_2 and TXB_2 from human aortic endothelial cells (Eder K et al, 2003) through the inhibition of PLA_2 activity, reduction of AA and mRNA concentration of PLA_2 and COX2. Both *c*9,*t*11 and *t*10,*c*12 CLA also reduced the production of PGE_2 in macrophages via the reduction of mRAN levels of COX2 (Yu et al, 2002).

1-C-2-4. Anti-inflammation

Eicosanoids play a pivotal role in inflammation as chemical mediators. CLA acts

as an anti-inflammatory agent through reducing the production of eicosanoids and interference with PPAR-γ and NF-κB (Lawrence et al, 2002).

The first mechanism by which CLA reduces the production of eicosanoids is that CLA can inhibit the activity of PLA_2 which is responsible for catalyzing the hydrolysis of ester bonds to release fatty acids from the sn-2 position of membrane phospholipids (e.g., AA or LA). A study of macrophage culture (Stachowska et al, 2007a) indicated that *c*9,*t*11- and *t*10,*c*12 CLA reduce the total activity of $cPLA_2$ and $sPLA_2$. *t*10,*c*12 CLA also reduces expression of $sPLA_2$ mRNA. The production of AA-derived eicosanoids is then reduced because the production of AA is reduced as the lowered availability of substrate for eicosanoids.

Secondly, during the metabolism of CLA, similar to LA, it undergoes desaturation via Δ6 desaturase to form conjugated diene (CD) 18:3 and further elongation and desaturation via Δ5 desaturase to form CD 20:3 and CD 20:4. CLA competes for these enzymes with LA, reducing the amount of AA formed (Banni, 2002). Furthermore, not only *c*9,*t*11 and *t*10,*c*12 CLA, but their metabolites also can be incorporated into neutral lipid (CD 18:3 and CD 20:3) and phospholipids (CD 20:4) in the same manner as LA, reducing the proportion of LA in tissue. Consequently, CLA competes with AA for incorporation into phospholipids, potentially modifying subsequent eicosanoid production (Banni et al, 2001). However, *t*10,*c*12 CLA generates larger amounts of CD18:3, but less amounts of CD20:3 compared with c9,t11 CLA due to difficulties in elongation of 6 *cis*, 10 *trans*, 12 *cis* 18:3. Therefore, *c*9,*t*11 CLA and its metabolites have a higher concentration in phospholipids than *t*10,*c*12 CLA (Ip et al, 1991).

Finally, CLA interferes with enzymes involved in AA metabolism. As indicated above (Ringseis et al, 2006; Yu et al, 2002 and Eder et al, 2003), through the activation of PPAR-γ, PPAR-γ-dependent inhibition of NF-κB, inhibition of PLA_2 and reduction of AA during the progression of atherosclerosis, CLA reduces the mRNA levels of COX1, COX2, PGIS, and mPGES and reduces the production of eicosanoids, such as PGE_2, PGI_2 and TXB_2 from human aortic endothelial cells, smooth muscle cells and macrophages. NF-κB is a key regulator for the transcription of pro-inflammatory molecules, such as cytokines including TNF-α, IL-1 β and IL-6, chemokines, such as MCP-1 and enzymes, including iNOS and COX2. The activated form of NF-κB is a heterodimer composed of a p50 and a p65 subunit which resides in the cytoplasm in an inactive form bound to the inhibitory protein, IκB (Chen et al, 2001). PPAR-γ can bind to and stabilize the inhibitory protein complex on the promoter of inflammatory genes (e.g. inducible nitric oxide synthase 2 or $iNOS_2$) which suppresses transcription in the absence of inflammatory signals. CLA can activate PPAR-γ as a ligand to maintain the repression of inflammation (Varga and Nagy, 2008).

Several studies have demonstrated that CLA also interferes with enzymes in the LOX pathway; however, the mechanisms are not yet clear. The possible mechanisms include the competition for available substrate, altering the mRNA and protein levels of the enzymes, or inhibiting activity of COX or LOX. A human cell culture study (Ochoa et al, 2004) revealed that *c*9,*t*11 CLA reduces 5-LOX protein and mRNA levels as well as COX1 and COX2 in prostate cancer. Investigation (Kim et al, 2005) of human breast tumor cells elucidated that both *t*10,*c*12 CLA and *c*9,*t*11CLA reduced the production of 5-HETE

through competing for substrate with AA, and *t*10,*c*12 CLA also decreased the FLAP mRNA levels. A human macrophage study (Stachowska et al, 2007b) found that both *t*10,*c*12 CLA and *c*9, *t*11 CLA inhibited the enzyme activity of 15-LOX-1 as an inhibitor. However, in isolated rat pulmonary cells (Cho et al, 2005), *c*9,*t*11 CLA inhibited the enzyme activity of 15-LOX-1 through competition with LA as substrate for the enzyme.

1-C-2-5. Anti-carcinogenesis

Several studies have suggested that CLA can interfere with the growth of induced tumours. In a mouse study carried out by Ha et al (1987), they reported that the application of CLA prior to 7, 12-dimethylbenz anthracene (DMBA) application in the two-stage mouse epidermal carcinogenesis system resulted in only about half as many papillomas as that in the control group. This result indicates that CLA can inhibit the initiation of the induced epidermal carcinogenesis in mice partially through the inhibition of CYP 450 activity and competing with linoleic acid during the AA metabolism. Furthermore, another study conducted by Belury et al (1996) in mice fed CLA mixture during skin tumour promotion induced by 12-O-tetradecanoylphorbol-13-acetate observed a reduction of papilloma incidence. It demonstrates that CLA inhibits tumour promotion in a manner that is independent of its anti-initiation activity.

Tumours are a result of imbalances between cell differentiation, proliferation and apoptosis. Therefore, the mechanisms associated with the anti-carcinogenesis effect of CLA include the modulation of cell proliferation and apoptosis. It was reported that dietary CLA mixture increased levels of p16 and p27 proteins which reduce cell proliferation by blocking DNA synthesis and cell cycle proteins that regulate this process (Belury, 2002). A

great deal of evidence (Majumder et al, 2002; Park et al, 2004 and Ochoa et al, 2004) demonstrated that dietary CLA induces apoptosis in numerous tissues including mammary gland, liver, colon and adipose tissues through the reduction of Bcl-2 protein.

Investigation of CLA isomers in anti-carcinogenesis has shown that there are differential effects of *c*9,*t*11 and *t*10,*c*12 CLA on some tumour incidences. In methylnitrosourea-induced mammary tumours of rats, both *c*9,*t*11 CLA and *t*10,*c*12 CLA were effective in reducing the number of mammary tumours (Clement et al, 2002). Further investigation of human breast cancer MCF-7cells (Chujo et al, 2003) showed that CLA isomers inhibit tumour cell growth through different mechanisms including the inhibition of growth induced by insulin, estrogen, epidermal growth factor, and apoptosis. *t*10,*c*12 CLA was found to inhibit the tumour cell growth in colon, colorectal and gastric cell lines; however, *c*9,*t*11 CLA does not exert this inhibition in colon HT-29 and Caco-2 cells and MCF-7 breast tumour cell lines (Kelley et al, 2007). In contrast, several studies have found that *t*10,*c*12 CLA promotes small intestine cancer cell growth in mice with a mutation of the APC gene (Rajakangas et al, 2003), and mammary tumours in female mice with altered mammary epithelium erbB2 gene expression (Ip et al, 2007).

Accumulating evidence indicates that the metabolites of AA are implicated in carcinogenesis. An investigation conducted by Accioly et al (2008) in a study of colon cancer cell lines confirmed that there was an increased expression of COX2 and high levels of prostaglandins, particularly PGE_2 in 80% to 90% of colon carcinomas. In agreement with Accioly et al, Bortuzzo et al (1996) affirmed that PGs stimulate cell proliferation in HT-29 and HCT-15 human colon carcinoma cells, and they further concluded that

lipoxygenase products such as LTB_4, 12(S)-HETE and 15(S)-HETE might exert proliferative effects in colonic carcinogenesis. In addition to COX and LOX enzyme, CYP epoxygenases also are involved in human cancer. Jiang et al (Jiang et al, 2007) reported that in human cancer tissues and cancer cell lines, CYP2J2 epoxygenase is over-expressed, enhancing tumour growth and carcinoma cell proliferation, and preventing apoptosis of cancer cells. In a further in vitro study of MDA-MB-231, Tca-8113 and A549 human breast cancer cell lines, they demonstrated that over expression of CYP2J2 with increased production of EETs or addition of synthetic EETs induced the migration and invasion of cell lines.

As described above, CLA can decrease the production of eicosanoids through inhibiting the activity of PLA_2 (Stachowska et al, 2007a), competing with LA for desaturation and elongation enzymes (Banni, 2002) or competing with AA and LA for incorporation into phospholipids (Banni et al, 2001), thus reducing the proportion of LA and AA in tissues. Additionally, CLA can inhibit enzymes involved in eicosanoid synthesis, such as COX1, COX2, PGIS, mPGES (Yu et al, 2002; Ringseis et al, 2006; Eder et al, 2003 and Ochoa et al, 2004) through activation of PPARs and PLA_2 and PPAR-γ-dependent inhibition of NF-κB, thus, decreasing production of cytokines and cytokines-induced eicosanoid release. CLA also interferes with LOXs by different mechanisms (Ochoa et al, 2004; Kim et al, 2005; Stachowska et al, 2007b and Cho et al, 2005) as discussed above, such as competition for available substrate, altering expression of the enzymes, or inhibiting activity of LOX.

At least one study has shown a relationship between the reduction in AA-derived

eicosanoids by dietary CLA and decreased tumorigenesis in tissues. In PC-3 cell study, *c*9,*t*11 CLA, but not *t*10,*c*12 CLA inhibited the tumour cell growth partially through reducing the transcription of 5-LOX and COX2 expression (Ochoa et al, 2004). Nevertheless, in breast cancer cells, *t*10,*c*12 CLA, but not *c*9,*t*11 CLA inhibited cell growth and reduced the production of 5-HETE (Kim et al, 2005). In rat colonic mucosa, a mixture of dietary CLA isomers decreased cellular proliferation and induced apoptosis partially because of the reduction of PGE_2 levels (Park et al, 2004). In canine mammary cells (Wang et al, 2006), both *t*10,*c*12 CLA and *c*9,*t*11 CLA suppressed COX2 protein levels and PGE_2 levels which are associated with mammary tumour growth.

1-D. Summary

Several studies have demonstrated that eicosanoids are implicated in liver steatosis and kidney damage associated with obesity. For example, COX2, TXB_2 are increased, but PGI_2 is decreased in livers of obese rats (Cao et al, 2008). 5-LOX, LTB_4, 5-HETE (López-Parra et al, 2008) and 12/15-LOX and 12-HETE (Martinez-Clemente, et al, 2010) contribute to liver steatosis in mice. COX2 (Drury et al, 2009), TXB_2 and PGI_2 (Okumura et al, 2000 and Aparajita et al, 2004) and 12/15-LOX (Xu et al, 2005) are increased, but COX1 (Komers et al, 2005) and PGE_2 (Aparajita et al, 2004) are decreased in kidney of *fa/fa rats*.

Many studies have confirmed that CLA decreases the production of eicosanoids by different mechanisms (Stachowska et al, 2007a; Ringseis et al, 2006; Yu et al, 2002; Eder et al, 2003; Ochoa et al, 2004; Kim et al, 2005; Stachowska et al, 2007b; Cho et al, 2005; and Banni et al, 2001). However, no in vivo studies have examined the alteration of multiple eicosanoids simultaneously in liver and kidney. Nor have the effects of specific

CLA isomers on overall profile of eicosanoids been reported.

1-E. Hypotheses

- Dietary CLA reduces the levels of eicosanoids in kidney and liver.
- Dietary CLA inhibits the activity and protein levels of enzymes involved in eicosanoid synthesis.
- Because *c*9,*t*11 and *t*10,*c*12 CLA have different effects on eicosanoid metabolism, NF-κB and PPAR-γ, the effects of CLA isomers will be isomer-specific.

1-F. Objectives

A previous study conducted in Dr. Taylor and our laboratories demonstrated that dietary *t*10,*c1*2 CLA compared to *c*9,*t*11 CLA or unsupplemented obese rats resulted in a reduction in liver fat content, but worsened renal histological damage (unpublished observations). Therefore, in the current study, lipidomic analysis of tissues from these rats was performed to examine the effect of CLA isomers on multiple eicosanoids. These tissues were obtained from 17 wk old male lean and *fa/fa* rats fed control diet and *fa/fa* Zucker rats fed control diet plus 0.4% (w/w) *c*9,*t*11 or *t*10,*c*12 CLA for 8 wk. The objectives were to determine:

- How CLA affects the endogenous levels and in vitro production of eicosanoids in livers and kidneys.
- Whether CLA affects the protein levels of enzymes involved in eicosanoid metabolism.
- Whether these effects of CLA are isomer- specific.

2. MATERIALS AND METHODS

2-1. Animal model and diet

Obese *fa/fa* rats have an autosomal-recessively transmmitted mutation of the *fa* gene encoding the leptin receptor. This results in obesity and hyperlipidemia, insulin resistance, hyperinsulinemia and liver steatosis (Ogawa et al, 1995). Obesity is noted as early as 3 wk of age and hyperlipidemia is found after weaning. As well as insulin resistance, the spontaneous evolution of liver steatosis is already present at 7 wk and does not get worse at 14 and 21 weeks (Forcheron et al, 2009). With respect to the development of renal damage, proteinuria is present by about 3 mth of age without any change in glomerular filtration rate (GFR). By 7 mth, proteinuria is marked and accompanied by segmental glomerulosclerosis and decreased GFR (Kasiske et al, 1992).

In the current study, the liver and left kidney tissues were collected from Zucker rats obtained from Dr. C Taylor's 2006 CLA study. These male *fa/fa* and lean Zucker rats were purchased from Harlan (Indianapolis, IN). The protocols for the animal care procedures were approved by the University of Manitoba Protocol Management and Review Committee and were within the guidelines of the Canadian Council on Animal Care. Rats were received at 16 wk and acclimatized for 5-7 days. During the acclimatization period, the rats were maintained in a controlled environment of 55% humidity, 21-23℃ within a 14 h : 10 h light : dark cycle, and fed a diet based on the AIN-93G diet without CLA.

After the adaptation period, ten rats at 17 wk of age were randomly assigned to each group. The four groups for the present study included a group of lean rats (lean

control) and a group of *fa/fa* rats given a control diet with no CLA (*fa/fa* control), a group of *fa/fa* rats given the control diet plus 0.4% (w/w) *c*9,*t*11 CLA (*fa/fa* 9,11 CLA) from a synthetic source (95% purity; Natural ASA, Hovdebygda, Norway) and a group of *fa/fa* rats given the control diet plus 0.4% (w/w) *t*10,*c*12 CLA (*fa/fa* 10,12 CLA) from a synthetic source (95% purity; Natural ASA, Hovdebygda, Norway). Diet formulations were based on the AIN-93G diet (Table 3) which constituted the control diet. This animal study lasted for 8 wk, during which time all rats consumed the diet ad libitum with fresh feed provided 3 times/wk.

2-2. Lyophilization of liver

Livers from each rat were lyophilized in preparation for analysis. Liver was removed from the -80℃ freezer and placed into a pre-weighed disposable plate covered with foil with holes. The plate containing liver was weighed again and the initial weight of the frozen liver was recorded. Plates then were put in a pre-cooled (-50℃) freeze dryer (Lyolab-300). All seals were greased so that the vacuum pressure was maintained from -4 to-10 millibar. The plates were removed from the freeze dryer and weighed every several hours or after an overnight drying period. When two consecutive equal weights were obtained, the freeze drying procedure was finished. Finally, the dried livers were pulverized and stored in -80℃. The left kidneys from these rats had been lyophilized

Table 3. Diet composition①

Ingredients (g/ kg)	**Control Diet**	***c*9,*t*11 CLA diet**	***t*10,*c*12 CLA diet**
Cornstarch②	363	363	363
Maltodextrin	132	132	132
Sucrose	100	100	100
Egg white	212.5	212.5	212.5
Cellulose	50	50	50
AIN-93G mineral Mix	35	35	35
AIN-93 vitamin Mix	10	10	10
Choline	2.5	2.5	2.5
Biotin mix③	10	10	10
Tert-butylhydroquinone②	0.014	0.014	0.014
Soy oil	85	70	70
***c*9,*t*11 CLA**	0	15	0
***t*10,*c*12 CLA**	0	0	15

① This diet formulation was based on AIN-93 G diet**.**

② Cornstarch was purchased from Castco Inc. (Etobicoke, Ontario);

Tert-butylhydroquinone was purchased from Sigma-Aldrich (St. Louis, Missouri); other ingredients were purchased from Harlan Teklad (Madison, Wisconsin)

③ Biotin mix contains 200 mg biotin/kg of cornstarch.

Table 4. Whole cell homogenization buffer for preparation of liver and kidney tissues for Western blotting

Ingredient [conc]	Amount	Final [conc]
500mM tris-HCL(6.1 g/100 mL) Fisher Scientific, BP154-1, Npean, Ontario	2 mL	50 mM
0.5M sucrose (17.2 g/100 mL) Sigma, S9378, Oakville, Ontario	10 mL	250 mM
200mM EDTA (pH=7.6, 0.67 g/10 mL), Sigma, ED4SS	200 µL	2 mM
100 mM EGTA (pH=7.5, 0.38 g/10 mL), Sigma, E4378	200 µL	1 mM
0.4M NaF (0.168 g/10 mL),Sigma, S6521	2.5 µL	50 µM
Deionized water	6.104 mL	
10% Triton X-100,Sigma, T8787	1 mL	0.5%
10 mM Na orthovanadate (1.839 mg/mL), Sigma, S6508	200 µL	100 µM
Aprotinin (2.5 mg/mL), Sigma, A1153	200 µL	25 µg/mL
Leupeptin (1 mg/mL), Sigma, L2884	500 µL	25 µg/mL
Pepstatin (2.5 mg/mL) In 90:10 methanol:glacial acetic acid, Sigma, P5318	200 µL	25 µg/mL
Soybean trypsin inhibitor (ST1) (1 mg/mL),Sigma, T9003	20 µL	1 µg/mL
50Mm 4-(2-aminnoethyl) benzene-sulfonylluoride (ABSF) (12 mg/mL), Sigma, A8456	57.6 µL	144 µM
2-mercaptoethanol,Fisher Scientific, BP176-100	14 µL	10 mM

previously by our lab using the same procedure.

2-3. Preparation of tissues for Western blotting

A whole cell fraction was isolated from tissues for Western blotting analysis using a buffer containing Triton X-100 and protease inhibitors (See Table 4 for details of ingredients). The buffer was prepared by mixing all ingredients, followed by sodium hydroxide (10 M NaOH, Fisher Scientific, BP358-212) to obtain a final pH of 7.2-7.4. For homogenization, 3 mL of buffer was added to 30 mg of lyophilized tissue in a glass, round-bottomed tube submerged in ice. The tissue in buffer was homogenized for 2×30 seconds using homogenizer (Polytron PT-MR2100, Kicematica AG, Luzernerstrasse, Switzerland) on a speed setting of 12. The resulting homogenate was poured into ultracentrifuge tubes and balanced before being inserted into a cold rotor (MAL-80) and spun at 33,000 rpm (100,000 g) for 35 minutes in a pre-cooled (4℃) ultracentrifuge (Opitmal MAX Ultracentrifuge, Beckman coulter, Inc. Canifornia, USA.) The whole cell fraction suspended in the resulting supernatant was transferred into 2 mL microcentrifuge tubes (Fisher Scientific) and stored at -80℃.

2-4. Total protein determination

The determination of total protein concentration was carried out using the Bradford method. As described in this method, protein forms a complex with the dye brilliant blue G causing the absorption maximum of the dye to shift from 465 to 595 nm. The amount of absorbance is proportional to the total amount of protein in the sample.

Protein standards in concentrations of 0.75, 0.5, 0.375, 0.25, 0.125, and 0.0625 mg/mL were made up with 2 mg/mL bovine serum albumin (BSA) (P0834, Sigma-Aldrich Co.)

stock solution and double-distilled H_2O (DDH_2O). Whole cell liver or kidney fractions (10X or 20X, respectively) were diluted with deionized water. A 96-well microplate (Corning Incorporated, Costar 3368, USA) was used, and wells were identified as blank only with DDH_2O, standard or sample. Ten μL of standard, diluted sample and blank were pipetted in triplicate, and 200 μL Bradford Reagent (Sigma, Cat. B6916) was added to each well using a multi-channel Eppendorf pipette. After incubation for 15 minutes at room temperature, the plate was read using a microplate reader (Fluostar Omega, BMG Ladtech GMBH, Ortenberg, Germany) to measure the absorbance of each well at a wavelength of 595 nm. During the measurement, the blank-corrected average was calculated by subtracting the average absorbance of the blank standard triplicate from the measurement of all other individual standard and unknown sample triplicates. Then, a standard curve was created by plotting the blank-corrected 595 nm average for each BSA standard, and the protein concentration of each unknown sample was calculated with this standard curve.

2-5. Western blotting

2-5-1. Optimization of antibody and protein amount

In an attempt to find the optimal concentration of primary antibody and the appropriate amount of protein to load on the gel, different concentrations of antibody based upon previous experience in our lab or the manufacture's recommendation were used to detect the bands of each target protein. Once optimized, a dose-response study was conducted with the optimal primary and secondary antibody concentration and different amounts of protein from samples from the fatty and lean groups to generate dose-response curves. The appropriate amount of total protein loaded in wells was based on these

dose-response curves. According to previous investigations in our lab, for all of proteins, a 1:20,000 dilution was optimal for the secondary antibody concentration in Western blotting analysis. Optimal concentrations, source of specific primary antibody and protein amount loaded for liver and kidney samples are summarized in Table 5. Representative blots and dose-response curves for each protein are presented in Figures 3-18.

2-5-2. Protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For the preparation of Western blotting, proteins from whole cell fractions were separated by gel electrophoresis. In SDS-PAGE, proteins with different molecular size are separated as proteins migrate differentially in an electrical field based primarily on size. Commercial 10% precast Tris-Glycine gels (Invitrogen, Cat. EC60485BOX, 1.5mm x 15 wells) or gel poured in the laboratory were used for all proteins. Gels were made using two glass plates, one long and one notched (GL PL, 27692,NCH&PL-3.99×4.18× 0.48) cleaned with 70% ethanol and placed with their cleaned sides together and two spacers (Hoefer T spacers, SE2619T-2-1.0 10.5 cm×1 cm×0.75 mm) for separating the 2 plates. The plates were lined up and screwed into a gel apparatus (Amersham Biosciences, part of Hoefer miniVE vertical electrophoresis system, 80-6418-77, Buckinghamshire, England). A 10% (See Table 6 for the details) gel solution was poured in the space between 2 plates using a Pasteur pipette until it reached the black line above the apparatus sponge. A small amount of 70% ethanol was then layered on top of the separating gel, and gel was left to polymerize for 45 minutes. After the gel had been polymerized the ethanol was removed with filter paper and a stacking gel solution (See Table 7 for the details) was poured on top

Table 5. Western blotting conditions for liver and kidney proteins①

Protein	**Primary antibody Concentration**		**Protein amount/well (μg)**		**Source of antibody （Cat.#, company）**
	liver	**kidney**	**liver**	**kidney**	**Liver and kidney**
COX1	1：250	1：250	16	20	160109, Cayman
COX2	1：250	1：250	14	14	160106, Cayman
TXBS	0②	1：200	14	14	160715, Cayman
12/15-LOX	1：1000	1：2000	8	8	sc32940, Santa Cruz
15-LOX-2	0②	1：500	14	14	sc67143, Santa Cruz

①Horseradish peroxidase conjugated secondary antibody dilution (Sigma, Cat. A0545) for all proteins was 1/20000

②Not used for this tissue

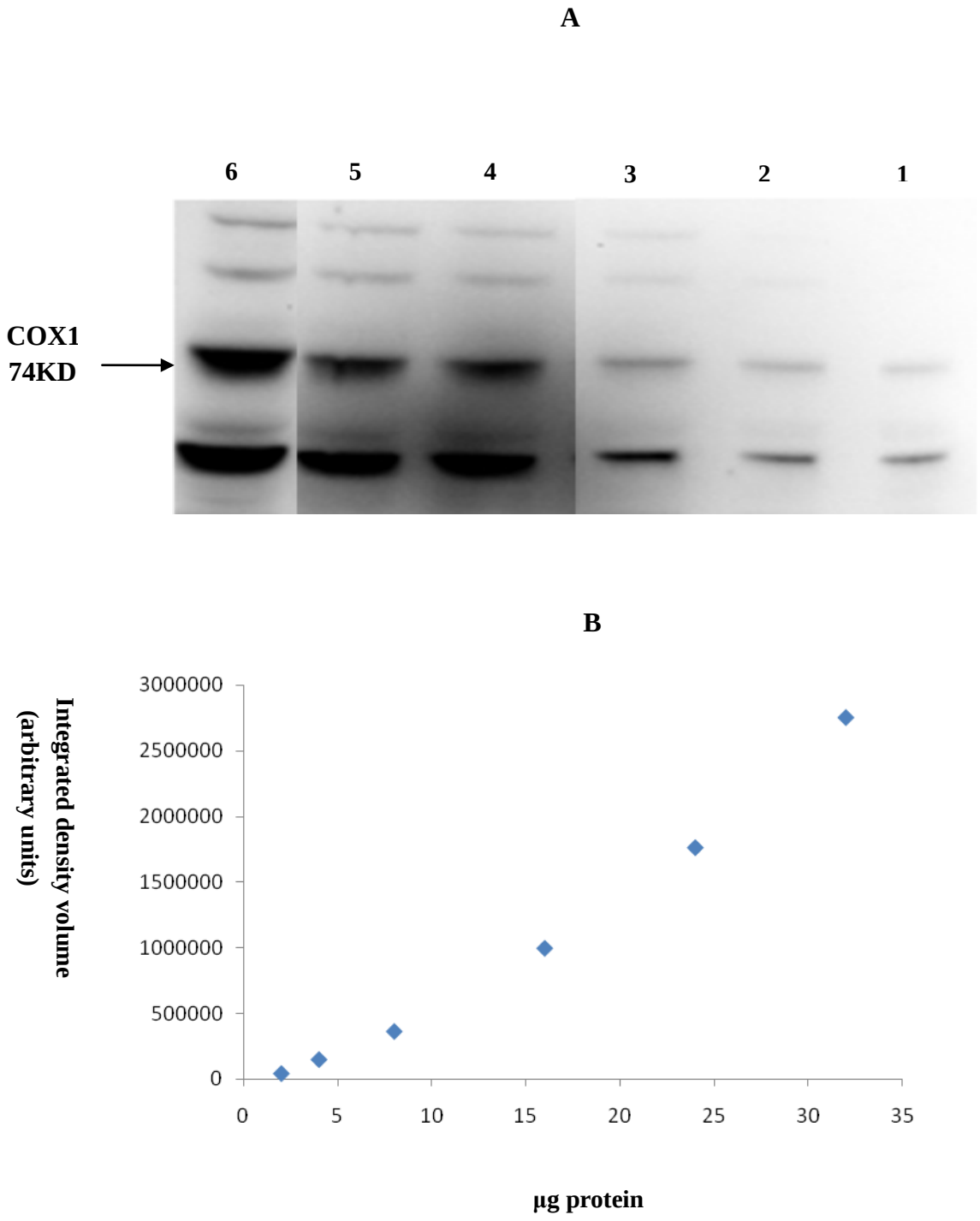


Figure 3. Western blot (A) and dose-response curve (B) for *fa/fa* rat liver COX1.

Arrow shows the bands of COX1. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

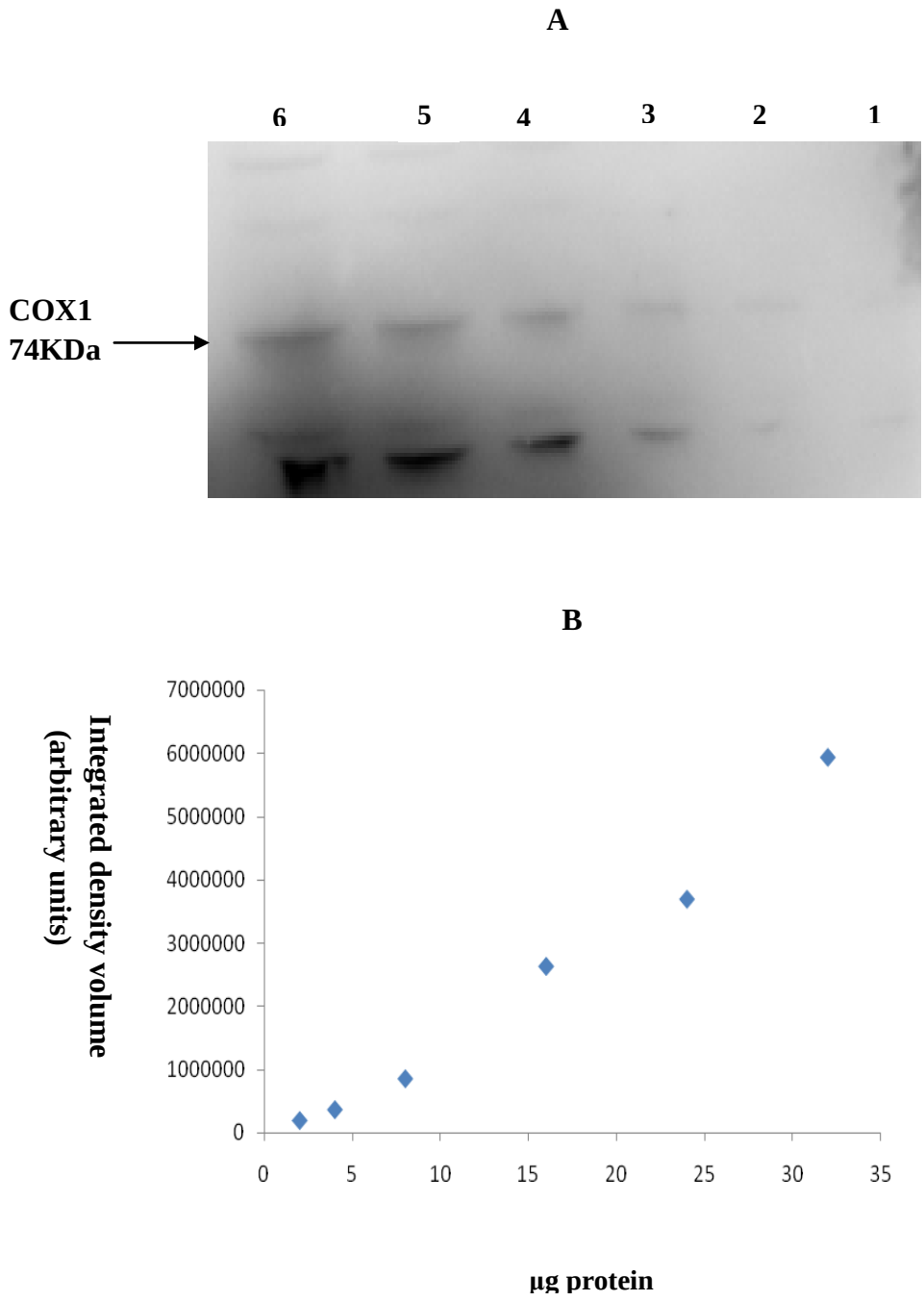


Figure 4. Western blot (A) and dose-response curve (B) for lean rat liver COX1.

Arrow shows the bands of COX1. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

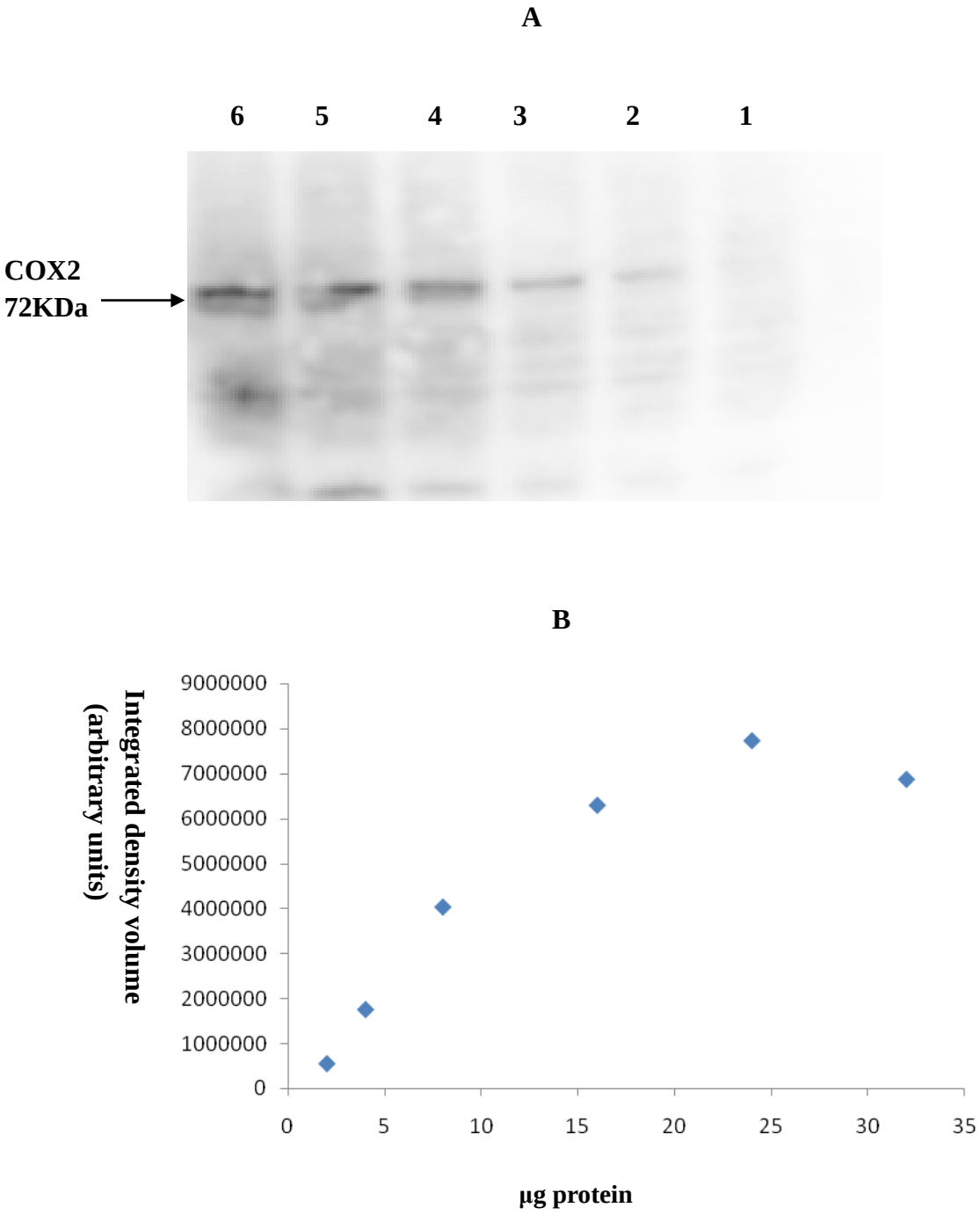


Figure 5. Western blot (A) and dose-response curve (B) for *fa/fa* rat liver COX2.

Arrow shows the bands of COX2. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

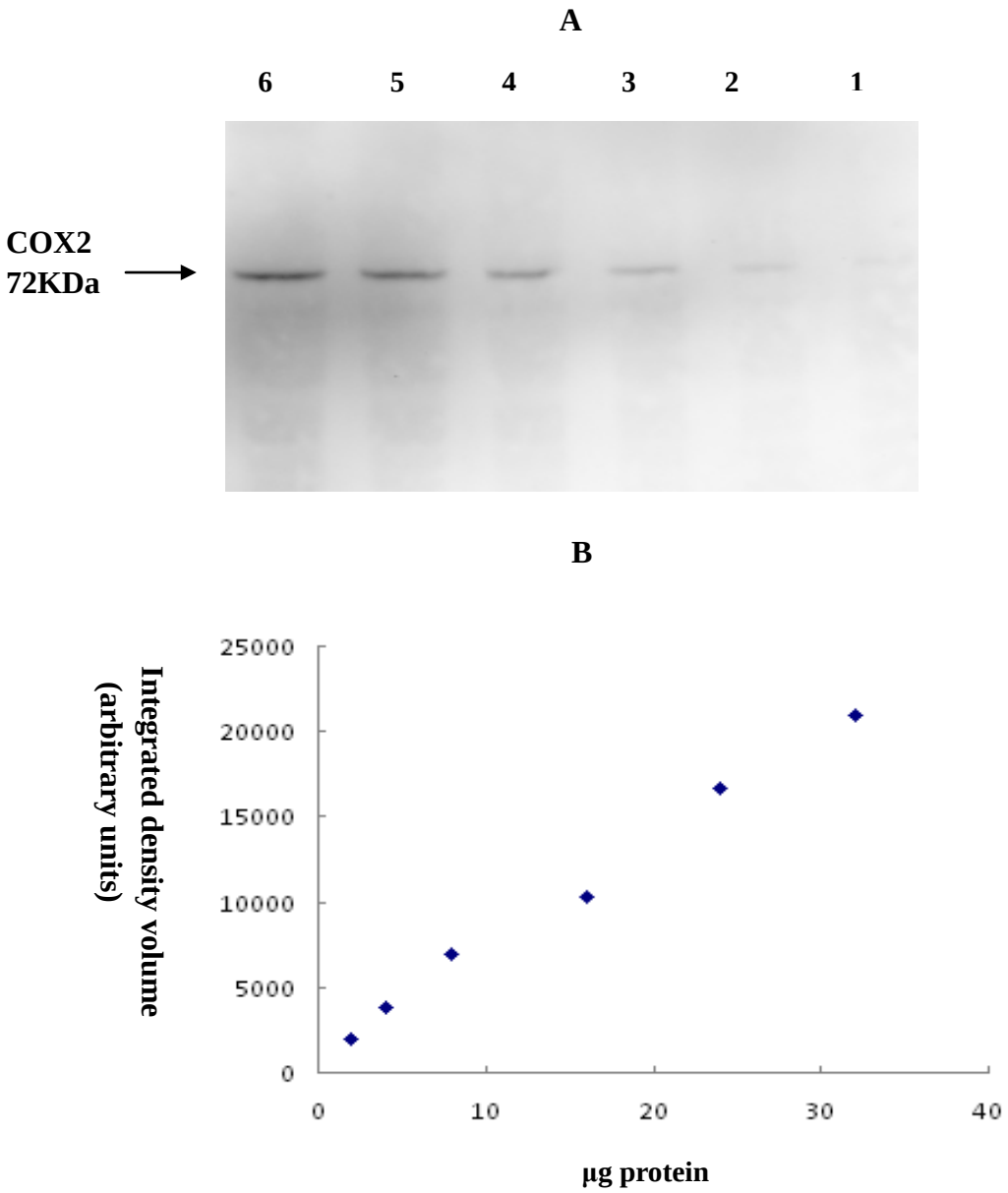


Figure 6. Western blot (A) and dose-response curve (B) for lean rat liver COX2.

Arrow shows the bands of COX2. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

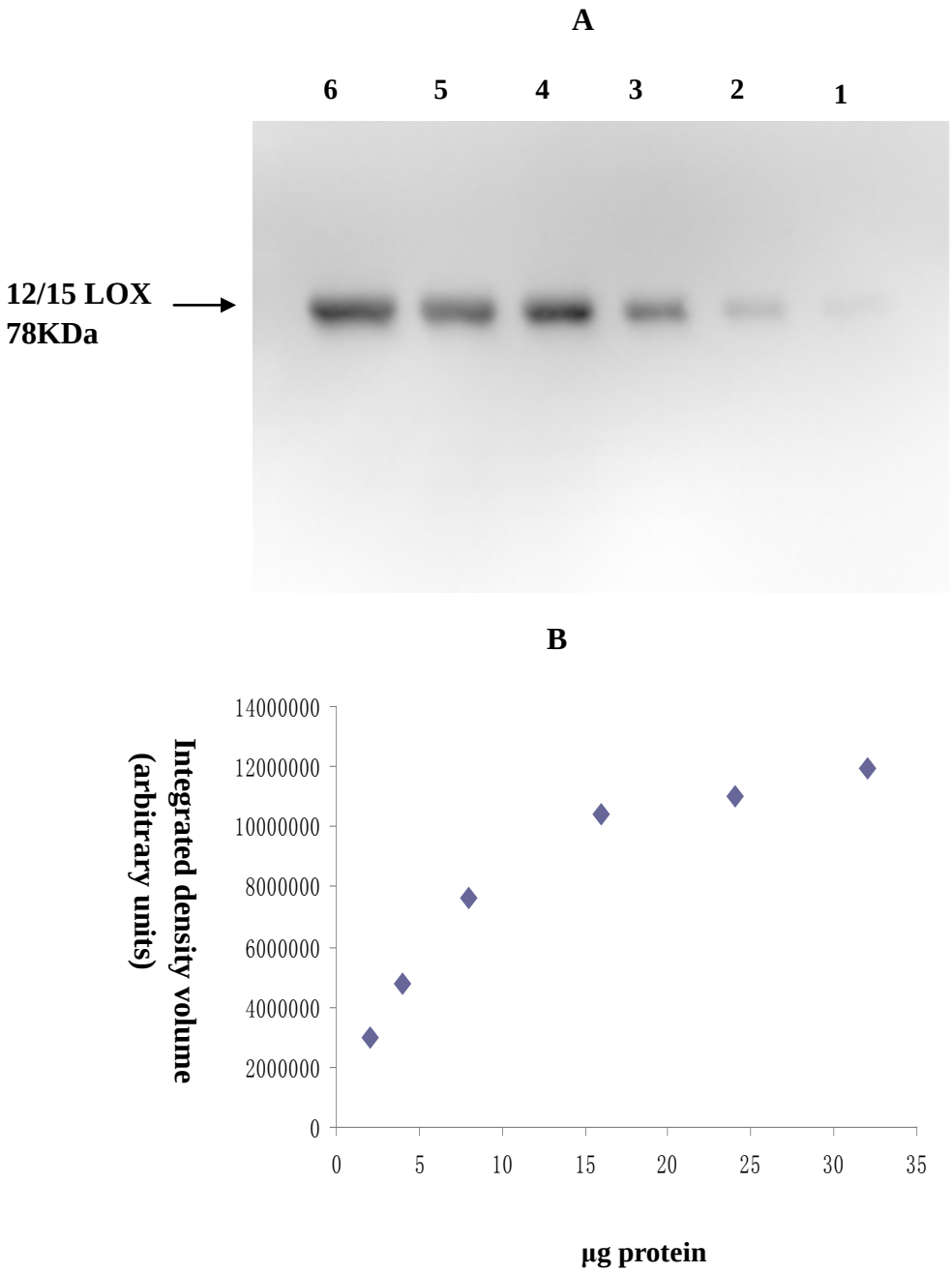


Figure 7. Western blot (A) and dose-response curve (B) for *fa/fa* rat liver 12/15-LOX.

Arrow shows the bands of 12/15 LOX. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

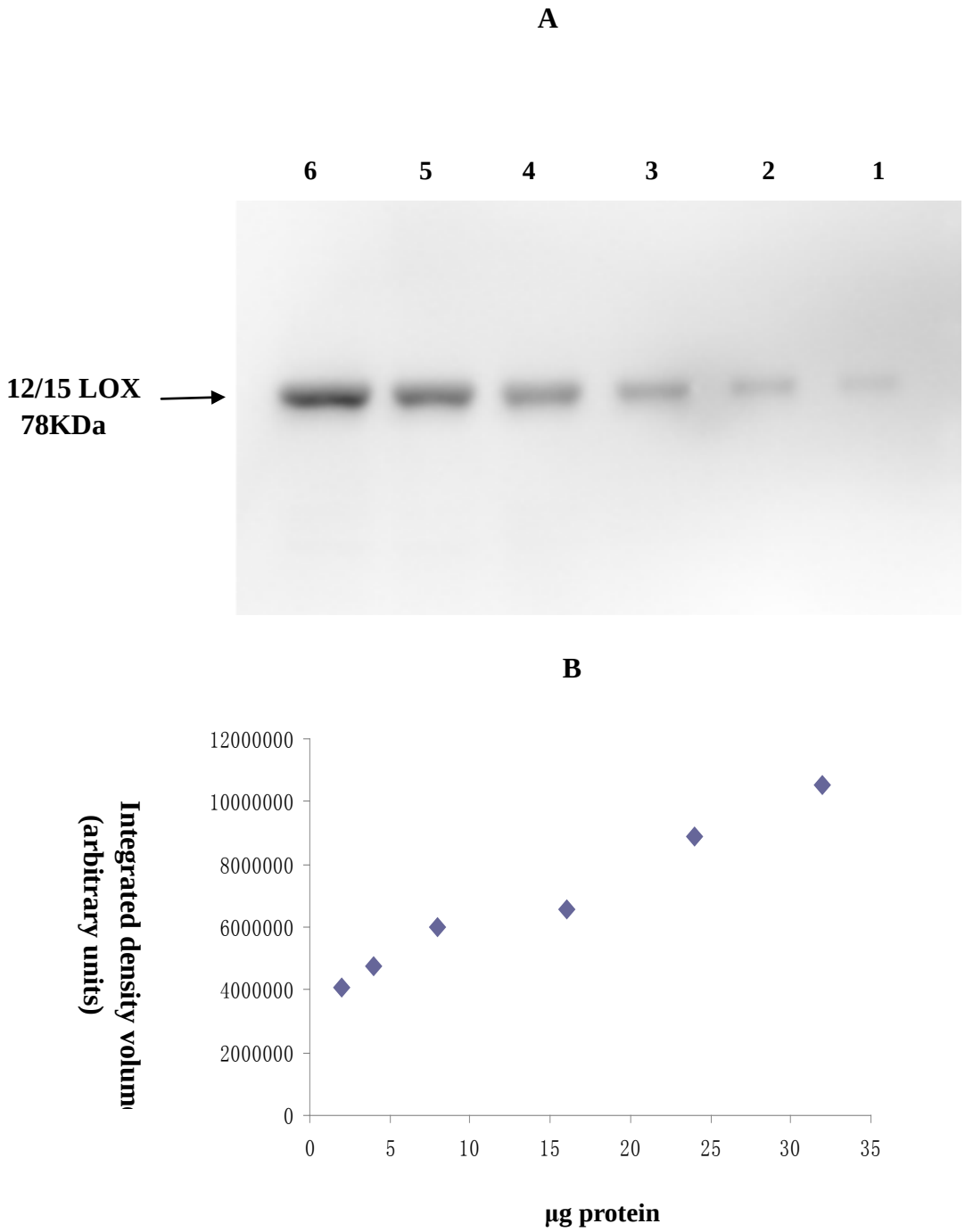


Figure 8. Western blot (A) and dose-response curve (B) for lean rat liver 12/15-LOX.

Arrow shows the bands of 12/15-LOX. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

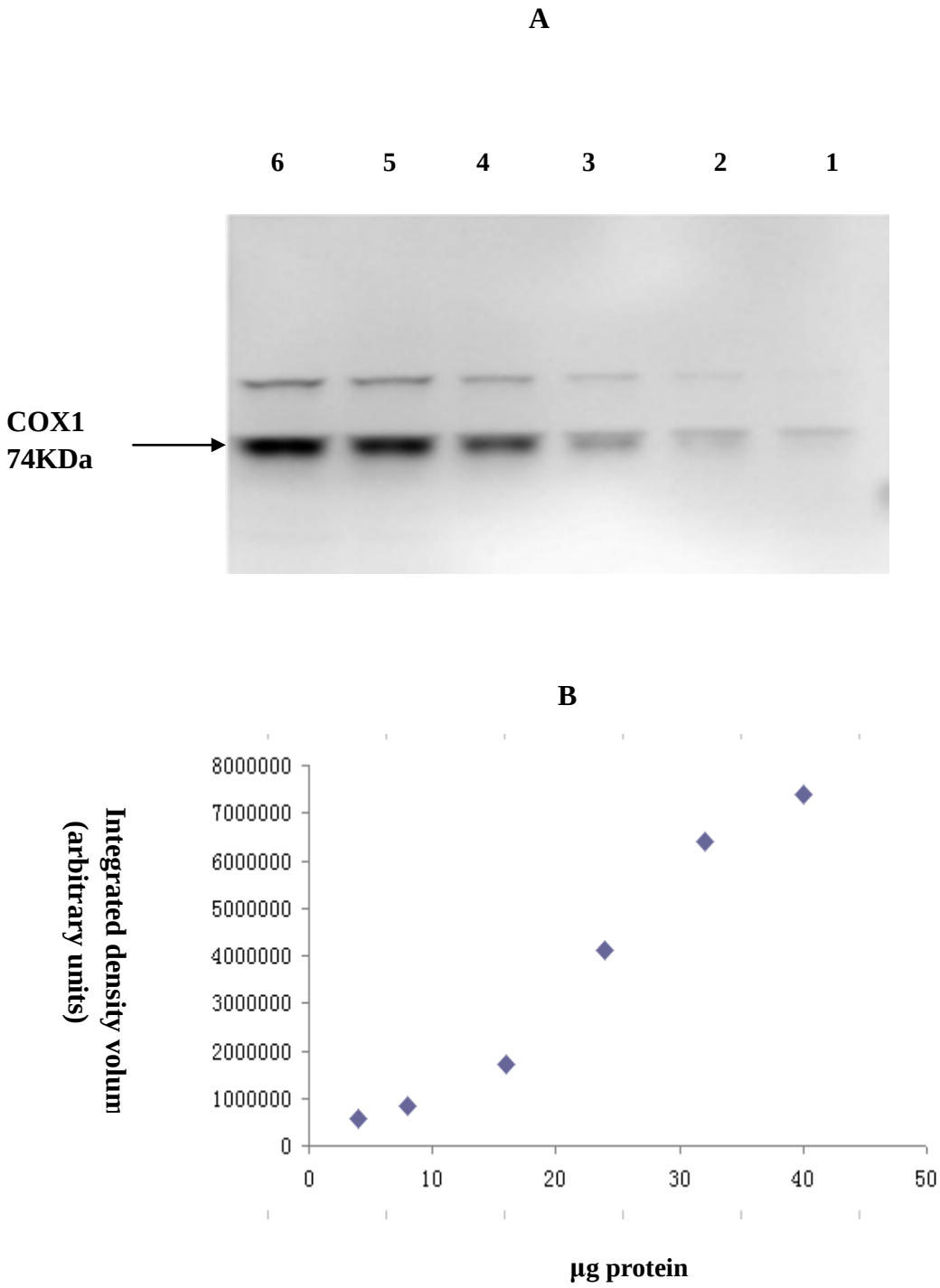


Figure 9. Western blot (A) and dose-response curve (B) for *fa/fa* rat kidney COX1.

Arrow shows the bands of COX1. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

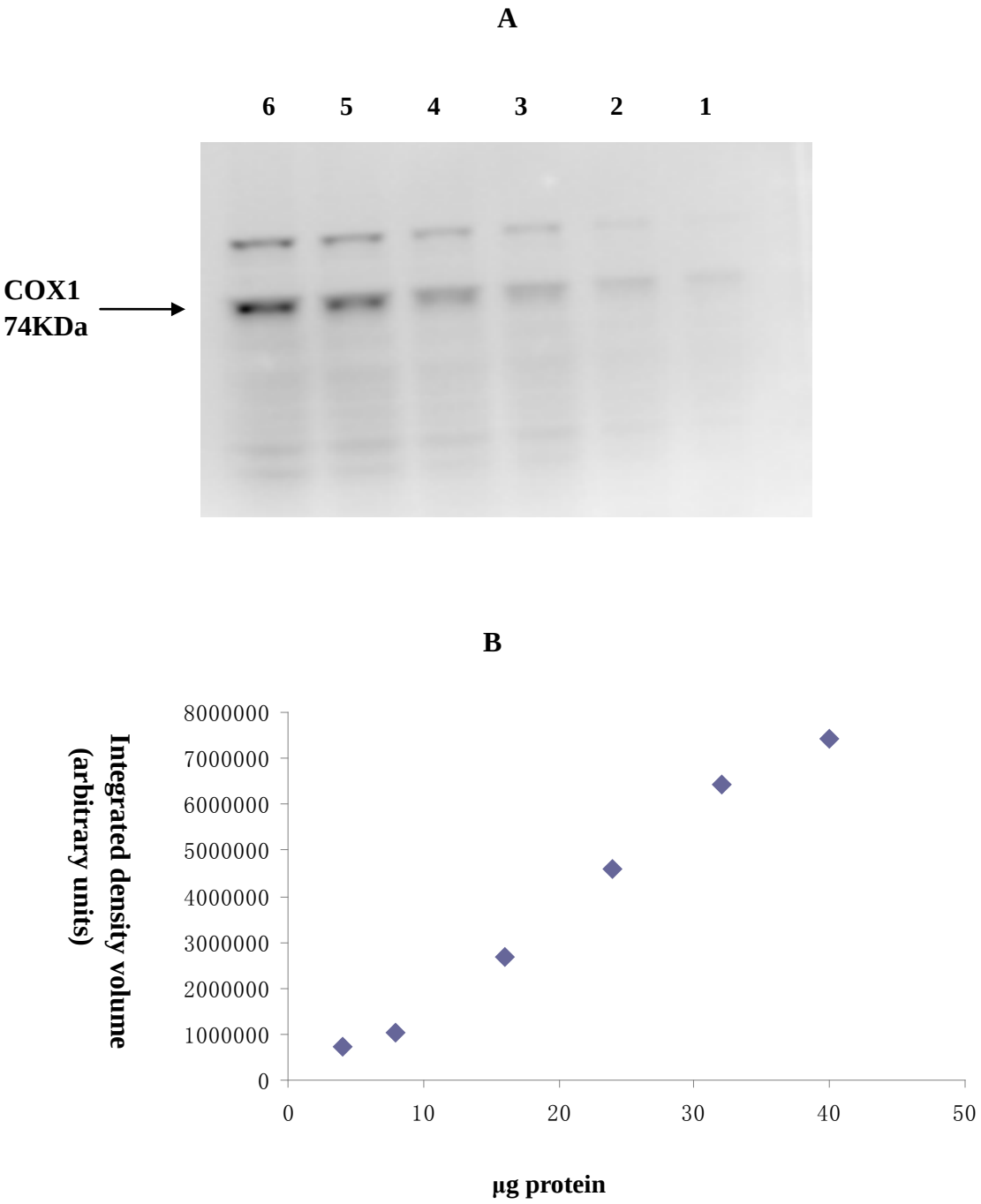


Figure 10. Western blot (A) and dose-response curve (B) for lean rat kidney COX1. Arrow shows the bands of COX1. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

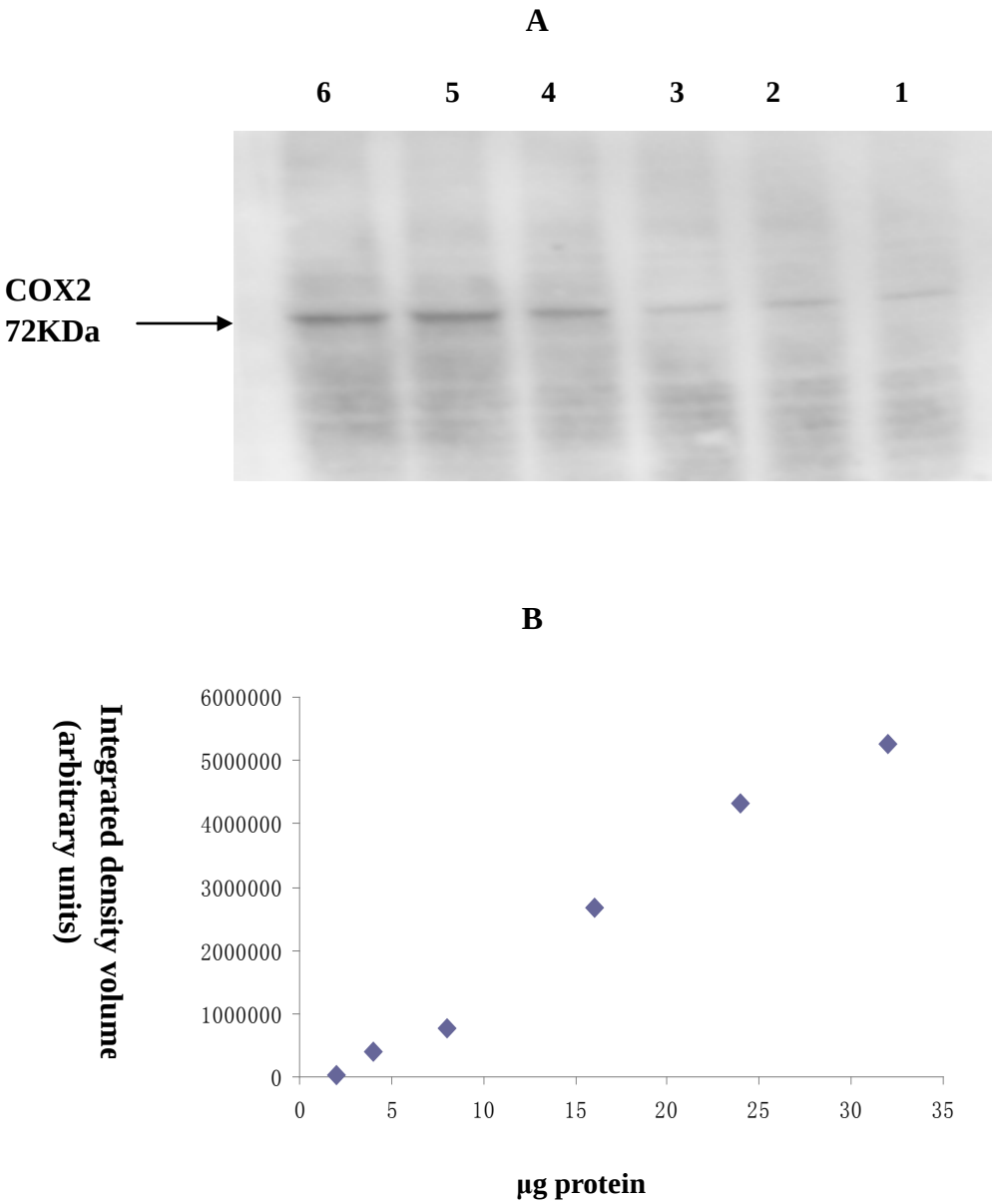


Figure 11. Western blot (A) and dose-response curve (B) for *fa/fa* rat kidney COX2.

Arrow shows the bands of COX2. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

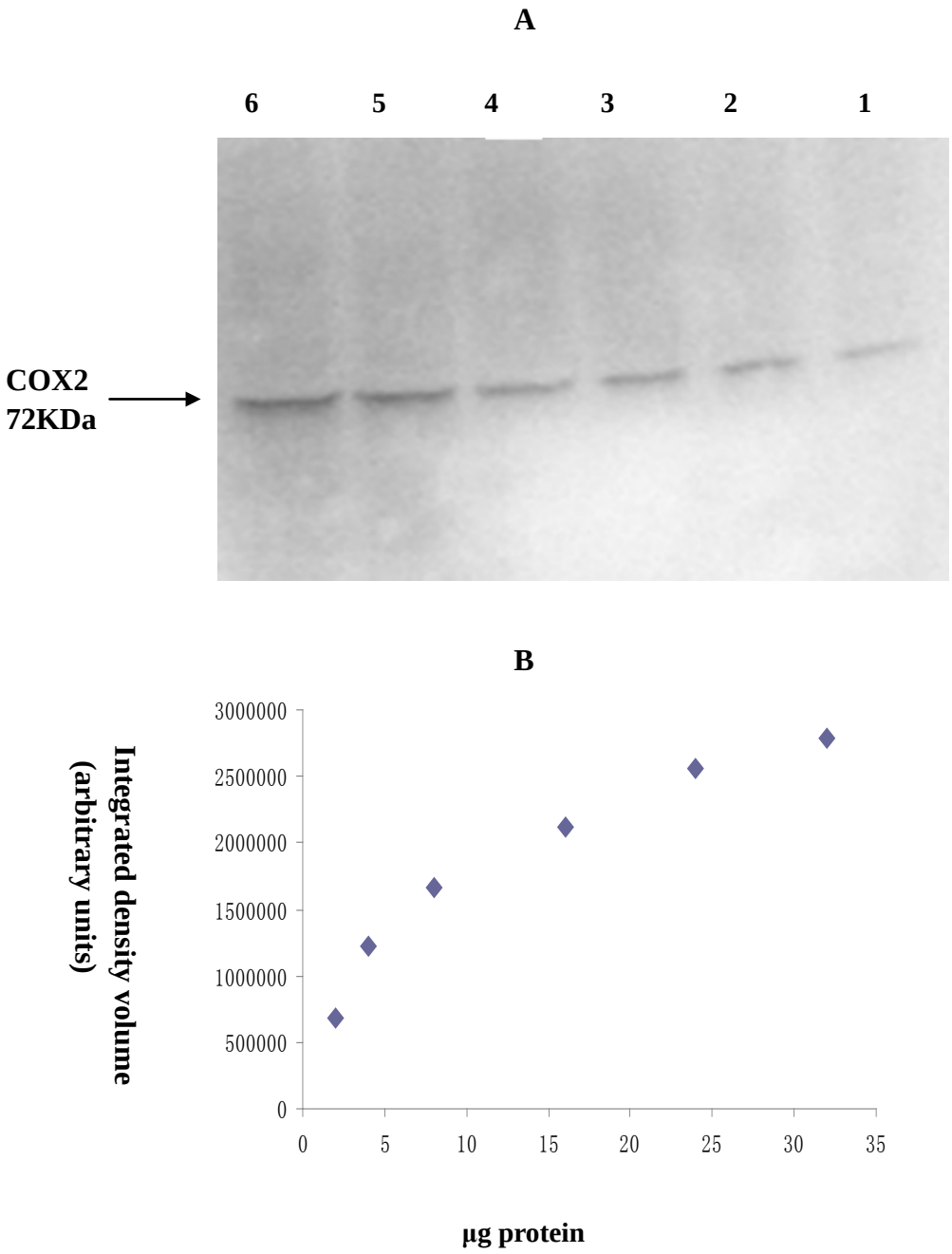


Figure 12. Western blot (A) and dose-response curve (B) for lean rat kidney COX2. Arrow shows the bands of COX2. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

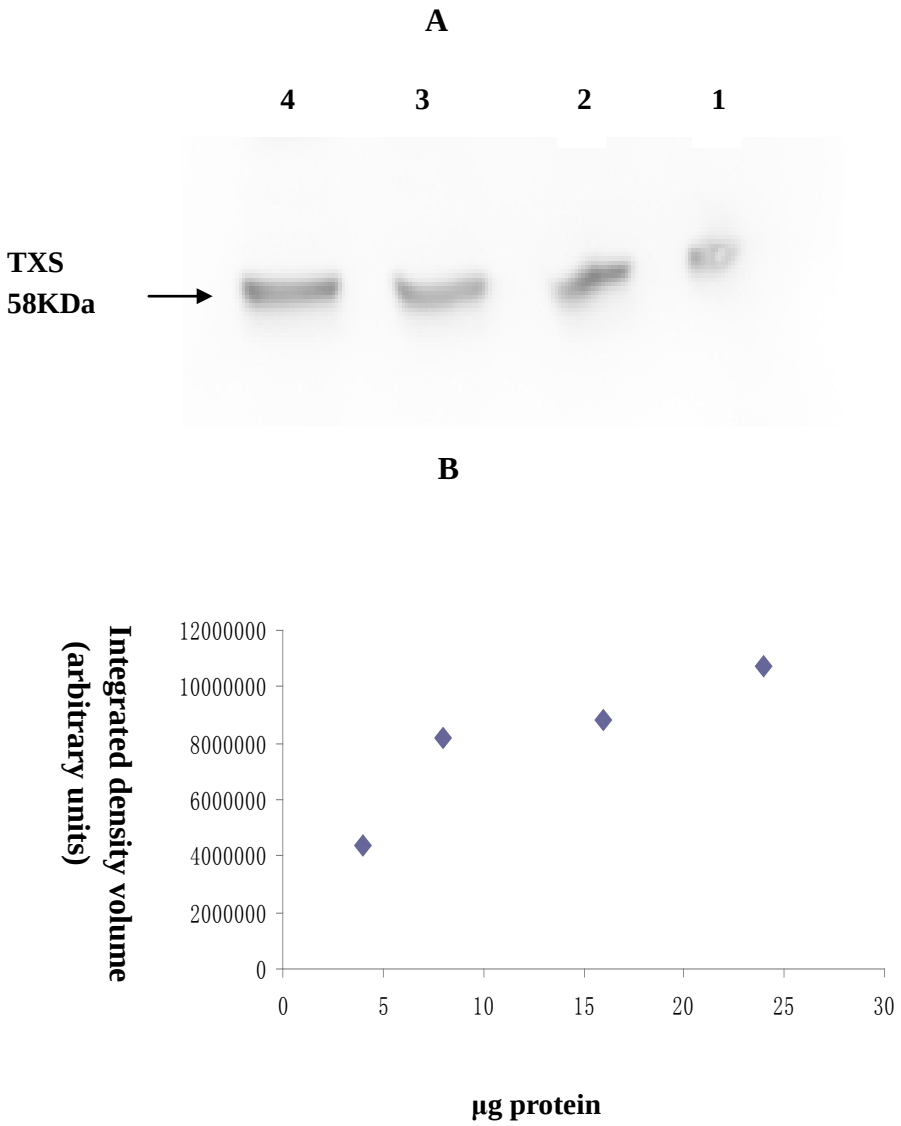


Figure 13. Western blot (A) and dose-response curve (B) for *fa/fa* rat kidney thromboxane A_2 synthase (TXS). Arrow shows the bands of TXS. The amount of protein loaded in the wells was 8, 16, 24, 32 µg from lane 1 to lane 4.

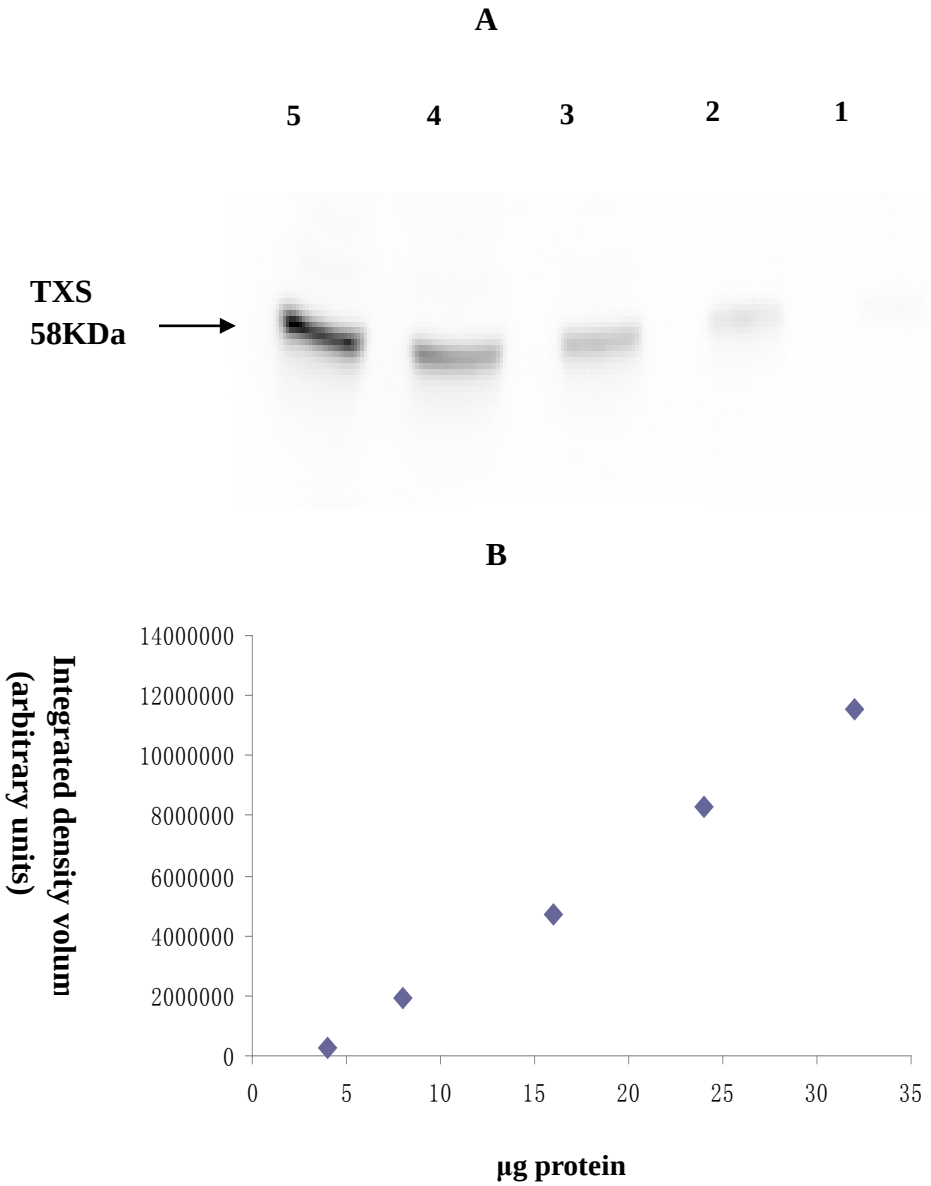


Figure 14. Western blot (A) and dose-response curve (B) for lean rat kidney TXS.

Arrow shows the bands of TXS. The amount of protein loaded in the wells was 4, 8, 16, 24, 32 µg from lane 1 to lane 5.

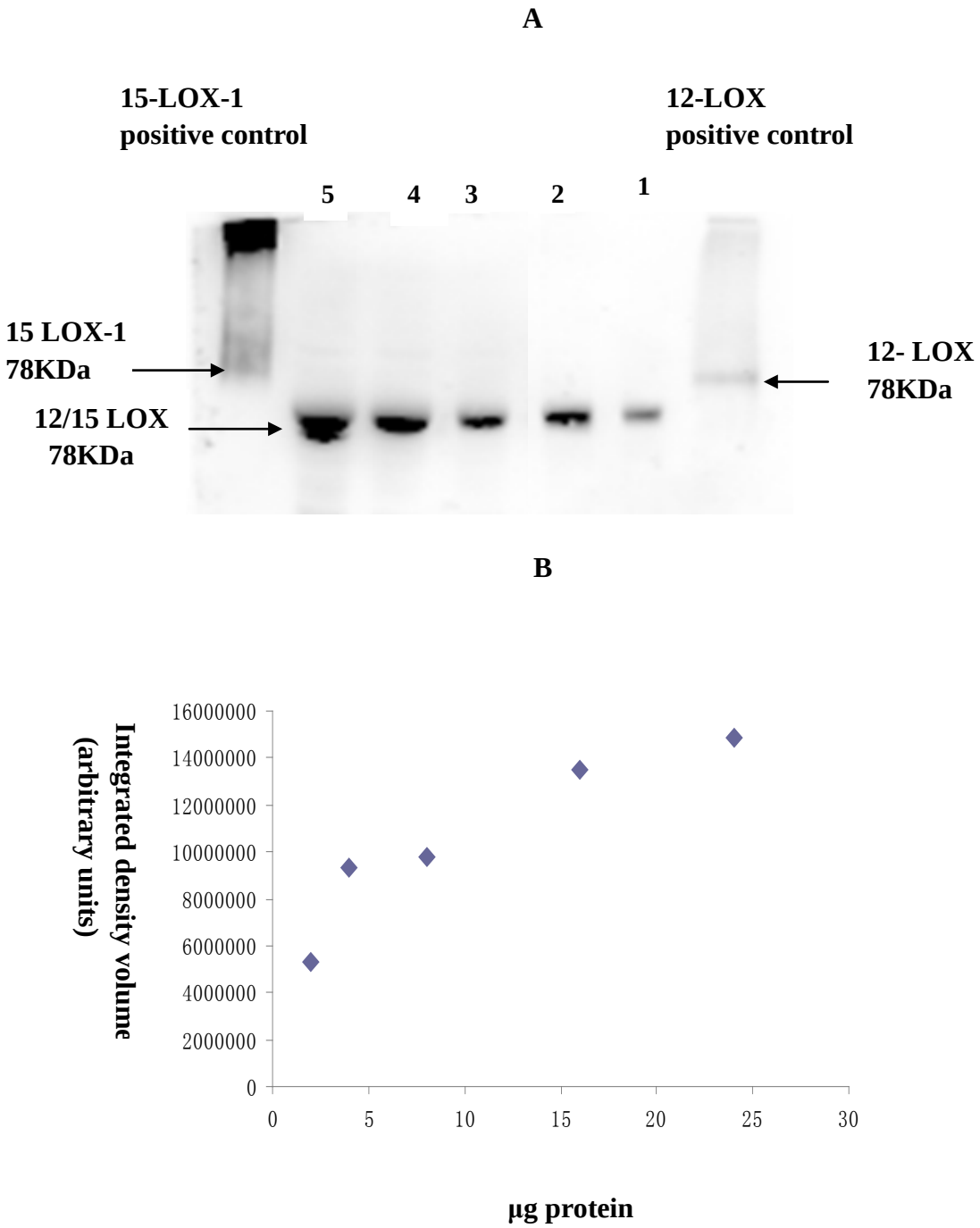


Figure 15. Western blot (A) and dose-response curve (B) for *fa/fa* rat kidney 12/15-LOX. Arrow shows the bands of 12/15-LOX. The amount of protein loaded in the wells was 2, 4, 8, 16, 24 µg from lane 1 to lane 5.

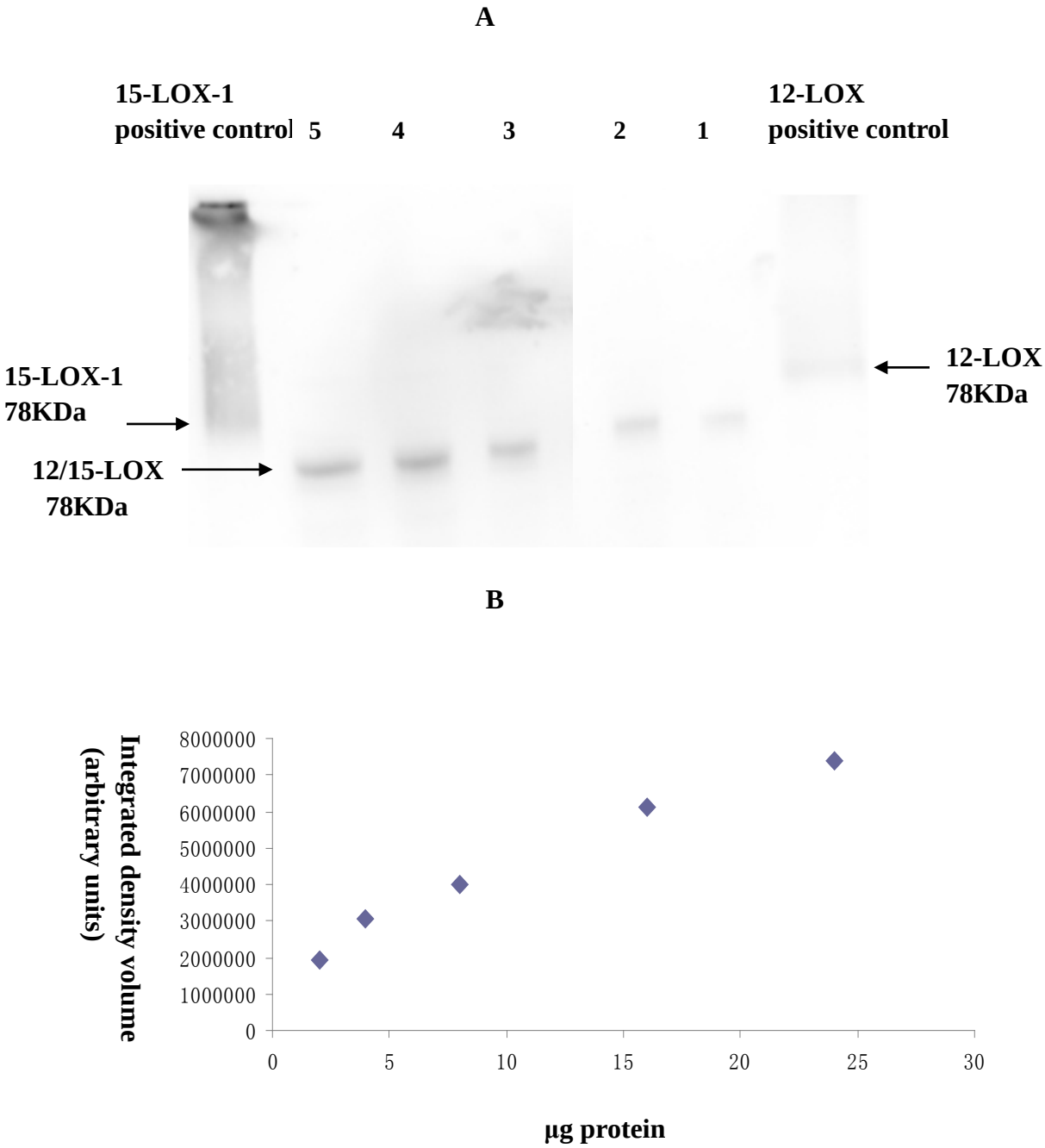


Figure 16. Western blot (A) and dose-response curve (B) for lean rat kidney 12/15-LOX. Arrow shows the bands of 12/15-LOX. The amount of protein loaded in the wells was 2, 4, 8, 16, 24 µg from lane 1 to lane 5.

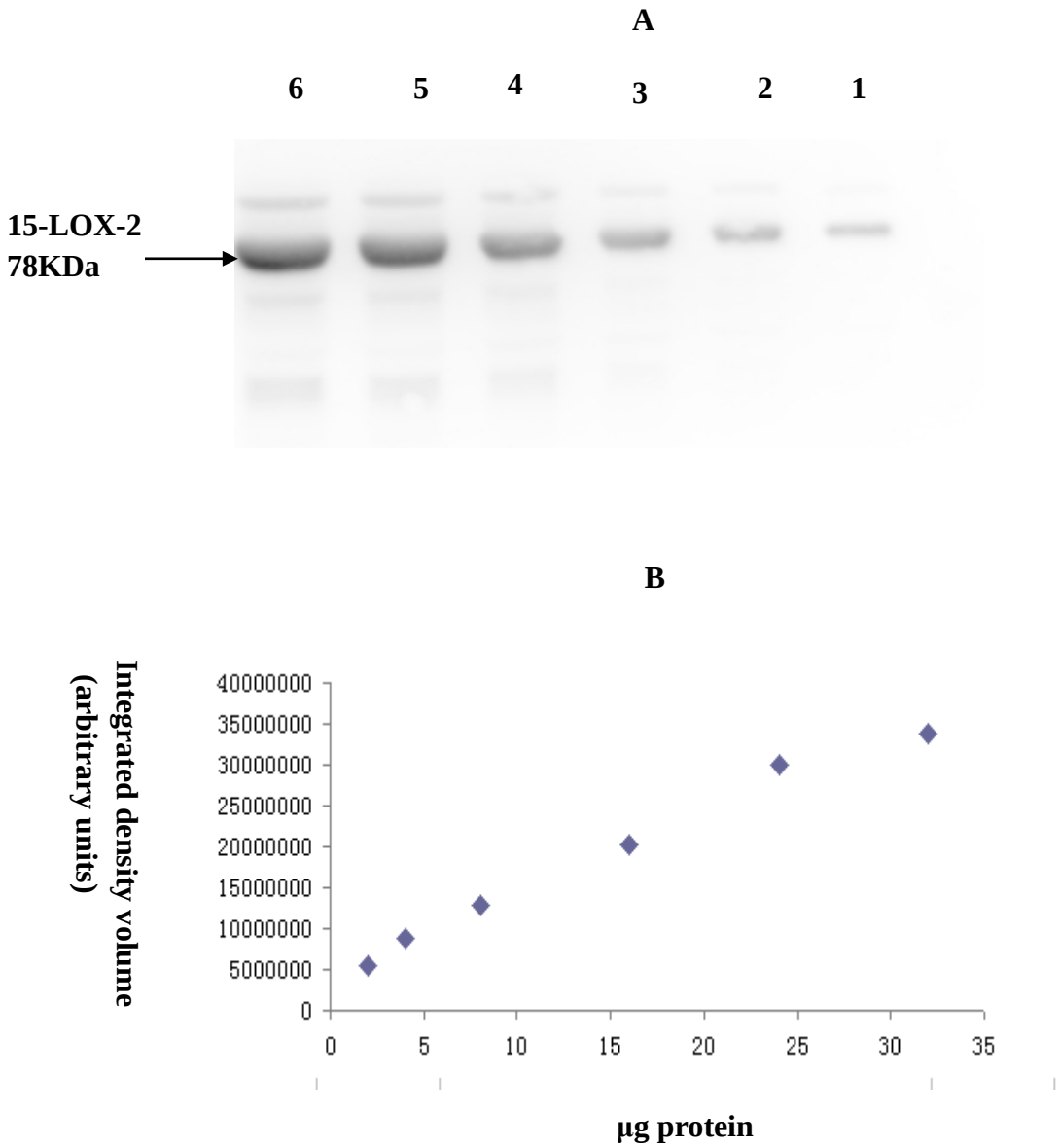


Figure 17. Western blot (A) and dose-response curve (B) for *fa/fa* rat kidney 15-LOX-2. Arrow shows the bands of 15-LOX-2. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

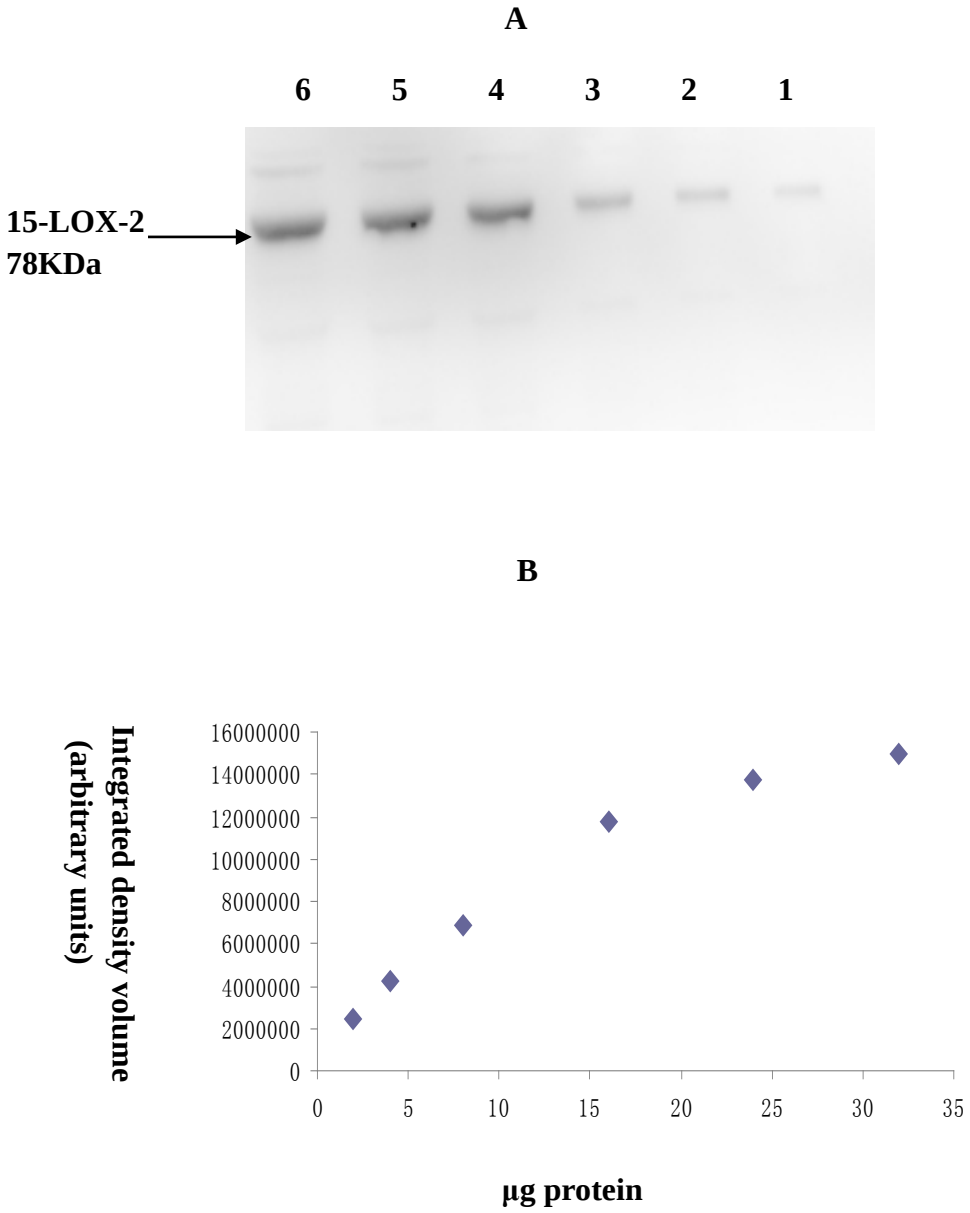


Figure 18. Western blot (A) and dose-response curve (B) for lean rat kidney 15-LOX-2. Arrow shows the bands of 15-LOX-2. The amount of protein loaded in the wells was 2, 4, 8, 16, 24, 32 µg from lane 1 to lane 6.

Table 6. Separating gel recipe for 2 gels for Western blotting

Reagent	**10% gel**
DDH$_2$O	4800 µL
1.5M Tris (Fisher Scientific BP 154-1)-HCl pH 8.8	2500 µL
10% (w/v) SDS (Fisher Scientific BP 166-500)	100 µL
Acrylamide/Bis 40% **(Fisher Scientific BP-1408-1)**	2500 µL
10% (w/v) Ammonium Persulfate (APS) **(Fisher Scientific BP 179-25)**	100 µL
Tetramethylethylenediamine (TEMED) **(Fisher Scientific BP-150-20)**	4 µL
TOTAL	10 mL

Table 7. Stacking gel recipe for Western blotting

Reagent	**Amount for 1 gel**
DDH_2O	1625 µL
1.5M Tris(Fisher Scientific BP 154-1)-HCl pH 6.8	625 µL
10% (w/v) SDS (Fisher Scientific BP 166-500)	25 µL
Acrylamide/Bis 40% (Fisher Scientific BP-1408-1)	250 µL
10% (w/v) Ammonium Persulfate (APS) **(Fisher Scientific BP 179-25)**	12.5 µL
Tetramethylethylenediamine (TEMED) **(Fisher Scientific BP-150-20)**	2.5 µL
Total	2.54 mL

of the separating gel with a Pasteur pipette. A comb was inserted into the stacking gel and set on the top of spacers to form lanes. After another 45 minutes of polymerization, the gel apparatus was placed in a chamber (Hoefer miniVe Complete, SE300-10A-1.0) filled with running buffer [100 mL DDH_2O + 900 mL 10X running buffer (29.0 g Tris Base + 144.0 g Glycine (Fisher Scientific BP381-1) + 10.0 g SDS) in 1 L DDH_2O with pH 8.3 adjusted with 1N HCL] in the center compartment and running buffer in the large chamber. This can be stored at 4℃ over night with the clamp bottom open.

Volumes of samples loaded into each well were calculated based on the total protein concentration determined by the protein assay. The equation was as follows: volume of sample (μL) [protein loaded in gel (μg) / protein concentration of sample (μg/μL)] + volume of DDH_2O (μL) [7 μL – volume of sample (μL)] + 7 μL of Laemmli 2X samples buffer (for samples with more than 2.0 μg/μL protein concentration). Then the relevant amount of protein extract was subjected to electrophoresis in the corresponding gel. Each sample, reference sample and 10 μL of Benchmark Prestained Protein Ladder (Invitrogen, Cat. 10748-010) were loaded in the wells. Electrophoresis was conducted at 125 V and the maximum of 400 mA for 1 hour 35 minutes.

2-5-3. Immunblotting

When the dye in the loading buffer ran to 0.5 cm from the bottom of the gel, a hydrophobic polyvinylidene fluoride (PVDF) membrane (GE Healthcare Amersham Hybond-P, Cat. RPN2020F) was cut to proper size and the left corner of the membrane was cut to indicate the first lane. The membrane was wetted with methanol (Fisher Scientific,

A452-4, Napan, Ontario) for 10 seconds, then immersed in DDH_2O on an orbital shaker. After 7-8 minutes, the membrane was put in 1X transfer buffer [3.03 g Tris base (Fish Scientific, Bp154-1), 14.4 g glycine (Fisher Scientific, Bp381-1) in mixture of 800 mL DDH_2O and 200 mL ethanol (Fisher Scientific, A452-4)] until the gel was ready for transfer.

After electrophoresis was finished, the gel module was removed from the chamber and the glass plates were separated. The stacking gel and the end of the separating gel with dye were cut off. The gel was then equilibrated in transfer buffer and placed in a blot module (Amersham Bioscience, 80-6418-96, Buckinghamshire, England) against the PVDF membrane, in between two pieces of filter paper with the cut corners matching up. Sponges were placed on both side of the gel/membrane to form a tight fit in the sandwich. The transfer apparatus was filled with 1X transfer buffer. The protein was transferred to the membrane at 24 V with a maximum of 400 mA for 1 hour and 45 minutes. Nonspecific binding sites on the membrane then were blocked with 5% nonfat milk (2.5 g milk in 50mL 1×TBS/Tween) for 1 hour. Ten ×TBS/Tween was made with 29.0 g tris base (Fisher Scientific, BP154-1, United States) and 80 g sodium chloride (Fisher Scientific, United States, BP358-212) in 800 mL DDH_2O, and then the pH was adjusted to 7.6 with 10 M HCl. Ten mL Tween (Fisher Scientific, BP337-500, United States) was then added. This was made up to 1 L with purified water to give a final Tween concentration of 0.1%. One hundred mL 10×TBS/Tween was diluted in 900 mL DDH_2O to make 1×TBS/Tween.

For immunoblotting, the membrane was probed with primary antibody which was prepared in 2% skim milk solution (0.3 g skim milk powder in 15 mL 1×TBS/Tween)

overnight at 4℃ on a rocker. The next morning the primary antibody was removed and the blot was washed 3 times in 1×TBS/Tween for 5-10 minutes, and then incubated with horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (Sigma, Cat. A-0545) at a concentration of 1:20,000 (0.75 μL in 15 mL1×TBS/Tween) for 1 hour at room temperature, and was washed again 3 times with 1×TBS/Tween for 5-10 minutes. To develop the blot, two chemiluminescent Peroxidase Substrates (Sigma, CPS160-1KT) (1:2 of reagent: buffer) were mixed 4-5 minutes before it was needed and evenly distributed on the blot. The protein of interest on the blot was detected by chemiluminescence with the Fluorochem imager (Alpha Innotech, Fluor Chem, Canifornia, USA) and the integrated density volume (IDV) was calculated and used as a measure of the intensity of the protein band. In order to get the IDV, an object box was placed around the protein band, large enough to encompass the entire band, but no larger than necessary. A background box was placed as close to the band as possible for getting a representative section of the background staining surrounding the protein band. These 2 boxes were then linked together so that the background pixels could be subtracted from the protein band pixels. Additionally, the reference bands from a reference standard which was made with a mixture of 100 μL of three samples from each group were applied to standardize the band intensities between gels, accounting for variation in background and gel density. The IDV of the protein sample was then divided by the average IDV of the 2 reference bands. The IDV was expressed in arbitrary units.

2-6. Measurement of eicosanoids

2-6-1. Homogenization and sample treatment

Tyrode's (pH 7.6) salt solution was made with 100 mL reconstituted Tyrode's salt [made with 1 L ultrapure water and 9.6 g Tyrode's salt (Sigma, T2145, Aldrich Canada LTD.)] and 100 mg powdered sodium bicarbonate, and adjusted to pH 7.6 with 10M HCL. This buffer was used to homogenize the dried liver or kidney tissue (for every 70 mg of dried tissue, 2000 μL of Tyrode's was used) using a homogenizer (Polytron PT-MR2100, Kicematica AG, Luzernerstrasse, Switzerland). After homogenization, 1/100 of homogenate volume of 1% Triton [made with 0.02 g triton (Triton X-100, T8787, USA) and 1.0 mL Tyrode's (pH 7.6)] was added to the homogenate to make a final concentration of Triton of 0.01% and placed on ice for 30 minutes. Two hundred μL aliquots were then taken for determination of endogenous eicosanoid levels (time 0) and enzyme activity measurements. To determinate the optimal incubation time for maximal eicosanoid production, time course studies were performed. In time course studies (Figure 19-23), the in vitro production of eicosanoids at 37℃ for 0, 5, 10, 20, 40, and 60 minute were determined in duplicate samples. According to the results of these time course studies, the most rapid production of eicosanoids was found to occur in the first 10 minutes of incubation in liver, and in the first 5 minutes in kidney. Consequently, we chose 0 and 10 and 0 and 5 minute incubation for liver and kidney, respectively, for analysis of enzyme activity. The remaining homogenate was stored for protein assay (using 10 X dilution as described in section 2-4) at -80℃. Eicosanoids were extracted and incubations were stopped with 500 mL methanol with 1% formic acid. 10 μL of anti-oxidant solution [made

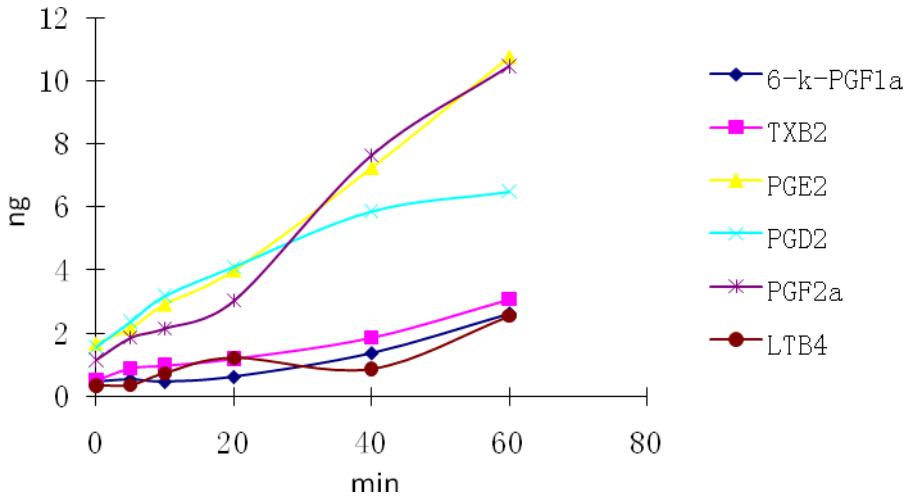


Figure 19. Time course of in vitro production of rat liver prostanoids and leukotriene B_4 (LTB_4). Data are averages of one sample from each of *fa/fa* 9,11 CLA, *fa/fa* 10,12 CLA, *fa/fa* control and lean control groups.

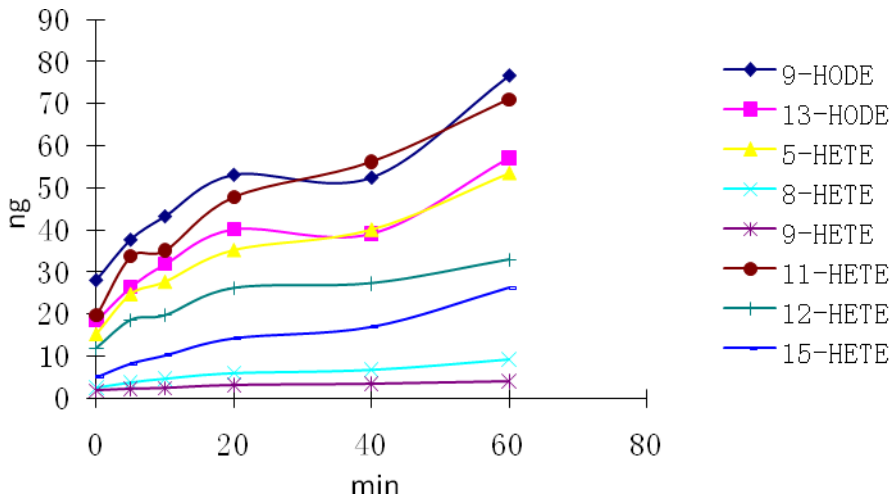


Figure 20. Time course of in vitro production of rat liver HODEs and hydroxyeicosatetraenoic acids (HETEs). Data are averages of one sample from each of *fa/fa* 9,11 CLA, *fa/fa* 10,12 CLA, *fa/fa* control and lean control groups.

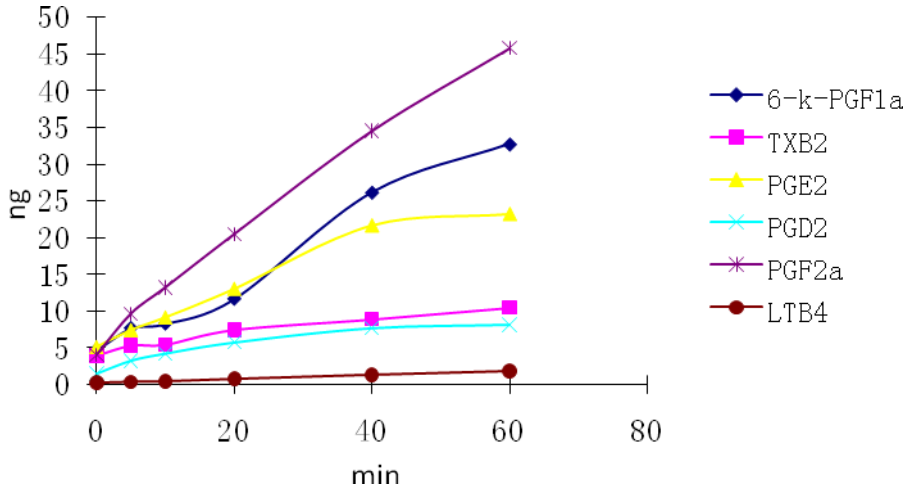


Figure 21. Time course of in vitro production of rat kidney prostanoids and LTB_4.

Data are averages of one sample from each of *fa/fa* 9,11 CLA, *fa/fa* 10,12 CLA, *fa/fa* control and lean control groups.

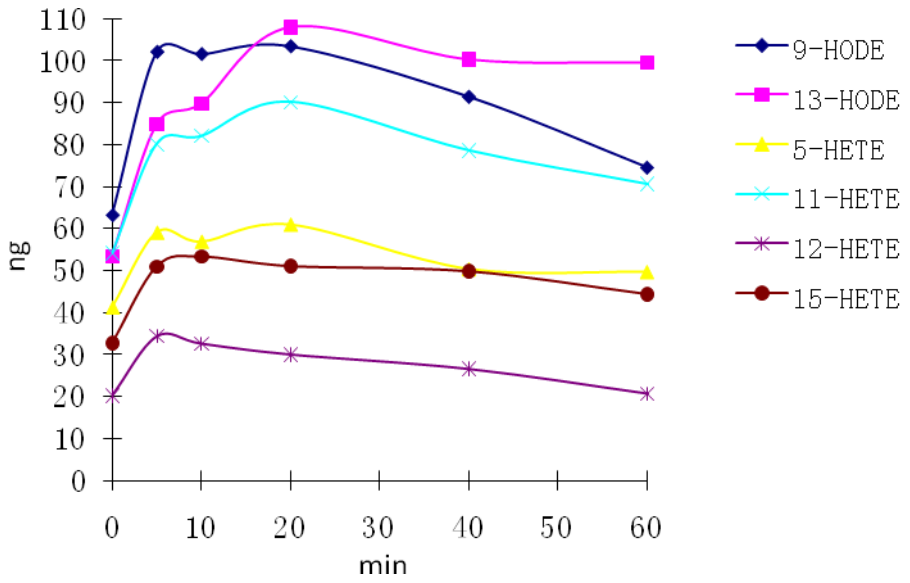


Figure 22. Time course of in vitro production of rat kidney HODEs and HETEs. Data are averages of one sample from each of *fa/fa* 9,11 CLA, *fa/fa* 10,12 CLA, *fa/fa* control and lean control groups.

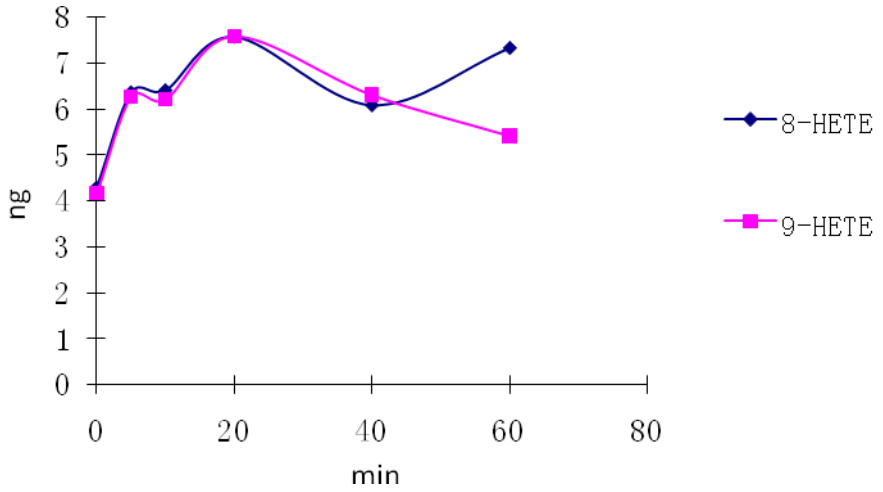


Figure 23. Time course of in vitro production of rat kidney 8- and 9- HETE. Data are averages of one sample from each of *fa/fa* 9,11 CLA, *fa/fa* 10,12 CLA, *fa/fa* control and lean control groups.

with 50 mL methanol, 24 mL ethanol, 25 mL ultrapure water, 1 mL 20 mg/mL butylated hydroxytoluene, 107.5 μL 0.5 M EDTA (Promega, V4233) and 200 mg Tri phenyl phosphite] also was added to stop in vitro formation of eicosanoids and prevent oxidation.

2-6-2. Solid-phase extraction of eicosanoids from tissues

Ninety μL 100% ethanol, 80 μL pH 3 H_2O, and 10 μL of an internal standard solution (see Table 8 and 9 for ingredients) were then added to all samples. Strata-X SPE (Solid-phase extraction) columns (Phenomenex Torrance, CA) were preconditioned with 2 mL 100% methanol followed by 2 mL pH 3 H_2O. Samples were acidified to pH 3 with 4 μL 1N HCL and centrifuged for 5 minutes at 3000 rpm at 4 ℃. After applying the supernatant to the column, 1 mL 10% methanol in pH 3 water was used to wash the sample tubes, and after centrifuging, the supernatant also was added to the column. The eicosanoids were then eluted with 1 mL 100% methanol. The eluant was dried under nitrogen in a 37℃ water bath and re-dissolved in 100 μL of solvent A (water-acetonitrile-formic acid [63:37:0.02 v/v/v] for liquid chromatography-mass spectrometry (LC-MS) analysis.

2-6-3. High performance liquid chromatography (HPLC) coupled to electrospray ionization (ESI) tandem mass spectrometry (MS)

Compared with GC-MS, ESI-MRM (electrospray ionization-multi-reaction monitoring) allows a large number of eicosanoids to be identified and quantitated directly from an aqueous sample in a single LC-MS/MS run without requiring derivitization. For the dectection of eicosanoids with HPLC−ESI-MS/MS: 1) HPLC separates the eicosanoids according to their chemical and physical characteristics. 2) ESI based collision-induced

Table 8. Deuterated internal standards used for liver samples

Internal standard	**Amount (µL)**	**Stock Conc (ng/µL)**	**Final Conc (ng/µL)**	**Analyte name**
6-k-PGF$_{1\alpha}$-d4	36.0	25	1.5	6-k-PGF$_{1\alpha}$
TXB$_2$-d4	36.0	25	1.5	TXB$_2$
PGF$_{2\alpha}$-d4	24.0	50	2.0	PGF$_{2\alpha}$
PGE$_2$-d4	24.0	50	2.0	PGE$_2$
PGD$_2$-d4	36.0	25	1.5	PGD$_2$
13,14-dh-15-k-PGF$_{2a}$-d4	24.0	50	2.0	13,14-dh-15-k-PGF$_{2a}$
LTB$_4$	48.0	25	2.0	LTB$_4$
20-HETE-d6	60.0	25	2.5	20-HETE
15-HETE-d8	48.0	25	2.0	15-, 12-HETE
5-HETE-d8	48.0	25	2.0	5-,11-,8-,9-HETE
13-HODE-d4	48.0	25	2.0	13-HODE
9-HODE-d4	48.0	25	2.0	9-HODE
Total	480.0			
Solvent (Ethanol)	120.0			
Desired Final Total	600.0			

Table 9. Deuterated internal standards used for kidney samples

Internal standard	**Amount (µL)**	**Stock Conc (ng/µL)**	**Final Conc (ng/µL)**	**Analyte name**
6-k-PGF$_{1\alpha}$-d4	24.0	25	1.5	6-k-PGF$_{1\alpha}$
TXB$_2$-d4	16.0	25	1.0	TXB$_2$
PGF$_{2\alpha}$-d4	8.0	50	1.0	PGF$_{2\alpha}$
PGE$_2$-d4	12.0	50	1.5	PGE$_2$
PGD$_2$-d4	24.0	25	1.5	PGD$_2$
13,14-dh-15-k-PGF$_{2a}$-d4	16.0	50	2.0	13,14-dh-15-k-PGF$_{2a}$
LTB$_4$	32.0	25	2.0	LTB$_4$
20-HETE-d6	40.0	25	2.5	20-HETE
15-HETE-d8	32.0	25	2.0	15-, 12-HETE
5-HETE-d8	32.0	25	2.0	5-,11-,8-,9-HETE
13-HODE-d4	32.0	25	2.0	13-HODE
9-HODE-d4	32.0	25	2.0	9-HODE
Total	300.0			
Solvent (Ethanol)	100.0			
Desired Final Total	400.0			

decomposition (CID) used in multi-reaction monitoring (MRM) mode produces characteristic precursor/product transitions to identify a large number of eicosanoids. 3) Deuterated eicosanoids are used as internal standards to accurately quantitate eicosanoids as well as accounting for losses during sample preparation. For each eicosanoid to be an internal standard which is chemically and structurally similar to the target analyte is selected (see table 8 and 9). According to the known concentration of each internal standard, the ratio of the unknown analyte peak area to internal standard peak area in the sample was used to calculate the amount of eicosanoid (Deems et al, 2007).

The HPLC (Agilent Technologies, Santa Clara, CA, USA) used was an Agilent 1100 Series HPLC System which was coupled to an API 2000 Triple Quadrupule Mass Spectrometer equipped with a Turbo Ion Source (ESI Source) (AB Sciex, Concord, ON). Separation of eicosanoids was performed by reverse-phase LC on a 250x2 mm, Luna 5u C18 (2) 100A column (00G-4252-B0). The mobile phase used was solvent A [mixture of water-acetonitrile-formic acid (70:30:0.02 v/v/v)] and solvent B [mixture of acetonitrile and isopropyl alcohol (50:50; v/v)]. After the column was equilibrated in solvent A, samples (dissolved in Solvent A) were injected using a 50 uL injection loop and eluted using the following gradient: 0% solvent B for 1 min; increase in solvent B to 25% between 1-3 min; increase in solvent B to 45% between 3-11 min; increase in solvent B to 60% between 11-13 min; increase in solvent B to 75% between 13-18 min; increase in solvent B to 90% from 18-18.5 min; increase in solvent B to 100% between 18.5-19 min and hold until 22 min; decrease in solvent B to 0% between 22-25 min.

MS analyses were conducted with MS API 2000 which was operated in MRM mode

and equipped with a Turbo Ion Source as an ESI Source. Turbo Ion Source was performed in negative electrospray mode. The amount of endogenous level of eicosanoids was expressed as the amount of analyte in the sample relative to protein quantity. The enzyme activity was calculated as the in vitro production of eicosanoids/min relative to protein quantity (in vitro production/min/mg protein).

2-7. Statistical analysis

Data were analyzed using one-way ANOVA with the statistical software program SAS (SAS Institute Inc., version 9.2). Data that were not normally distributed (9-HETE in liver) were log transformed. Those data that still were not normally distributed after being log transformed (COX1 in kidney) were tested using the Kruskall-Wallis test. The protected Least Squares Means difference test was conducted as a post-hoc analysis to detect differences between groups. Spearman's correlation was used to test the correlation between endogenous levels or in vitro production of prostanoids with COX1 levels in kidney. $p<0.05$ was considered statistically significant. Non-transformed data are reported in the results section as mean ± SE.

3. RESULTS

3-1. Endogenous levels and in vitro production of eicosanoids in liver

The endogenous levels and in vitro production of 14 eicosanoids detected in liver are shown in Table 10 and Table 11, respectively. As shown in Table 10 and Figure 24, the endogenous level of hepatic LTB_4 was not significantly different between lean and *fa/fa* control groups; however, *t*10,*c*12 CLA supplementation significantly increased LTB_4 compared with *fa/fa* controls ($p<0.01$), but this effect was not significant in the *c*9,*t*11 CLA supplemented group. Lean rats had higher levels of hepatic 9-HODE ($p<0.01$), 13-HODE ($p<0.05$) and total HODEs ($p<0.005$) than *fa/fa* rats (Figure 25). Neither CLA isomer affected the endogenous levels of 9-HODE or 13-HODE in *fa/fa* rats. In vitro production of heptic LTB_4 was not different in *fa/fa* rats compared to lean rats and CLA isomers did not alter this (Figure 24). Consistent with the endogenous levels, lean rats had higher in vitro production of 9-HODE ($p<0.01$), 13-HODE ($p<0.05$) and total HODEs ($p<0.05$) compared with *fa/fa* rats and CLA supplementation did not affect these (Figure 25). There were no significant differences in prostanoid or HETE levels or in vitro production of these eicosanoids due to diet or genotype.

3-2. Protein levels of COX1, COX2 and 12/15-LOX in liver

Protein levels of upstream enzymes in the prostanoid and HODE synthetic pathways were detected by Western blotting (Figure 26). The protein level of COX1 was not different between genotypes and CLA supplementation also did not affect its level. In contrast, *fa/fa* rats had higher protein levels of COX2 than the lean counterparts ($P<0.005$), and both *c*9,*t*11 CLA ($P<0.005$) and *t*10,*c*12 CLA ($P<0.05$) supplementation

Table 10. Endogenous levels of liver eicosanoids in lean and *fa/fa* rats given control diets and *fa/fa* rats given 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (ng/mg protein)

Analyte Name	lean control (n=7)	*fa/fa* control (n=7)	*fa/fa* 9,11 CLA (n=7)	*fa/fa*10,12 CLA (n=7)
6-k-PGF$_{1\alpha}$	0.13 ± 0.02	0.14 ± 0.01	0.14 ± 0.02	0.14 ± 0.02
TXB$_2$	0.20 ± 0.03	0.12 ± 0.02	0.15 ± 0.03	0.15 ± 0.03
PGF$_{2\alpha}$	0.78 ± 0.08	0.85 ± 0.11	0.85 ± 0.11	0.66 ± 0.09
PGE$_2$	0.68 ± 0.12	0.72 ± 0.08	0.71 ± 0.08	0.59 ± 0.06
PGD$_2$	0.88 ± 0.16	0.88 ± 0.09	0.79 ± 0.16	0.76±0.12
PGs	2.67 ± 0.39	2.71 ± 0.28	2.64 ± 0.33	2.30 ± 0.24
LTB$_4$	0.05 ± 0.01^{ab}	0.02 ± 0.005^{b}	0.07 ± 0.02^{ab}	0.12 ± 0.04^{a}
9-HODE	20.17 ± 4.08^{a}	9.35 ± 1.03^{b}	9.56 ± 1.04^{b}	8.64 ± 0.94^{b}
13-HODE	12.40 ± 2.76^{a}	6.57 ± 0.89^{b}	6.39 ± 0.64^{b}	6.28 ± 0.85^{b}
HODEs	32.57 ± 6.81^{a}	15.92 ± 1.90^{b}	15.96 ± 1.66^{b}	14.92 ± 1.74^{b}
5-HETE	5.95 ± 1.23	7.58 ± 1.25	8.49 ± 1.35	7.83 ± 1.21
9-HETE	0.83 ± 0.17	0.79 ± 0.11	0.95 ± 0.16	0.85 ± 0.10
8-HETE	1.35 ± 0.23	1.12 ± 0.15	1.16 ± 0.15	1.26 ± 0.11
11-HETE	11.55 ± 1.68	8.50 ± 0.76	9.37 ± 1.49	8.58 ± 0.35
12-HETE	3.14 ± 0.50	3.00 ± 0.17	3.94 ± 0.30	3.72 ± 0.45
15-HETE	2.17 ± 0.42	2.28 ± 0.31	2.29 ± 0.15	2.11 ± 0.19
HETEs	24.00 ± 3.98	23.26 ± 2.27	26.18 ± 2.75	24.35 ± 1.88

All data are expressed as mean ± SE. Values having different superscripts are significantly different from each other. P value <0.05 was considered statistically significant.

Table 11. In vitro production of liver eicosanoids in lean and *fa/fa* rats given control diets and *fa/fa* rats given 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (ng/min/mg protein)

Analyte Name	lean control (n=7)	*fa/fa* control (n=7)	*fa/fa* 9,11 CLA (n=7)	*fa/fa*10,12 CLA (n=7)
6-k-PGF$_{1\alpha}$	0.005 ± 0.001	0.005 ± 0.002	0.005 ± 0.001	0.006 ± 0.001
TXB$_2$	0.02 ± 0.003	0.03 ± 0.006	0.03 ± 0.005	0.02 ± 0.005
PGF2$_\alpha$	0.05 ± 0.005	0.11 ± 0.03	0.08 ± 0.020	0.05 ± 0.01
PGE$_2$	0.08 ± 0.02	0.14 ± 0.04	0.11 ± 0.02	0.06 ± 0.02
PGD$_2$	0.10 ± 0.04	0.15 ± 0.03	0.14 ± 0.02	0.09 ± 0.02
PGs	0.21 ± 0.06	0.43 ± 0.09	0.36 ± 0.06	0.22 ± 0.05
LTB$_4$	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.03 ± 0.01
9-HODE	2.00 ± 0.52^a	0.79 ± 0.14^b	0.92 ± 0.15^b	0.77 ± 0.15^b
13-HODE	1.58 ± 0.45^a	0.72 ± 0.13^b	0.83 ± 0.13^b	0.61 ± 0.11^b
HODEs	3.58 ± 0.97^a	1.51 ± 0.26^b	1.76 ± 0.28^b	1.38 ± 0.26^b
5-HETE	0.56 ± 0.09	0.68 ± 0.14	0.70 ± 0.13	0.66 ± 0.08
8-HETE	0.10 ± 0.03	0.09 ± 0.03	0.10 ± 0.03	0.11 ± 0.03
15-HETE	0.35 ± 0.08	0.31 ± 0.06	0.35 ± 0.06	0.24 ± 0.04
9-HETE	0.07 ± 0.01	0.07 ± 0.02	0.08 ± 0.01	0.07 ± 0.02
11-HETE	1.16 ± 0.22	0.86 ± 0.17	0.93 ± 0.14	0.80 ± 0.14
12-HETE	0.45 ± 0.07	0.49 ± 0.05	0.43 ± 0.07	0.43 ± 0.08
HETEs	2.69 ± 0.48	2.49 ± 0.44	2.58 ± 0.41	2.31 ± 0.35

All data are expressed as mean ± SE. Values having different superscripts are significantly different from each other. P value <0.05 was considered statistically significant.

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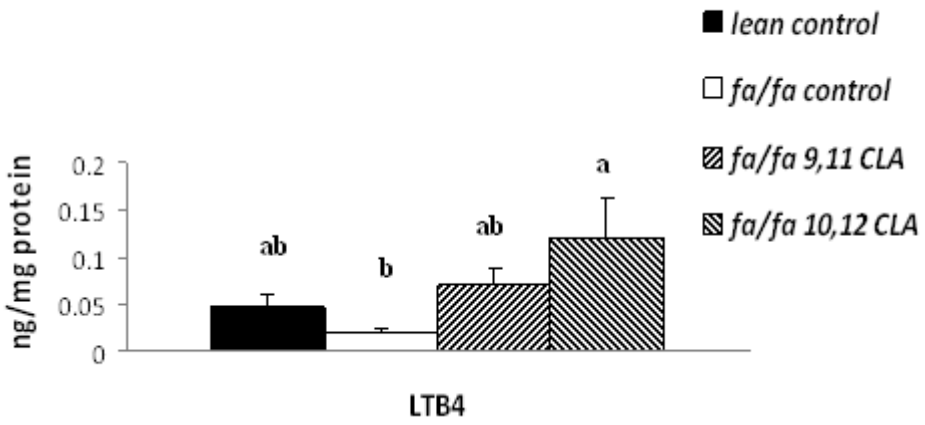

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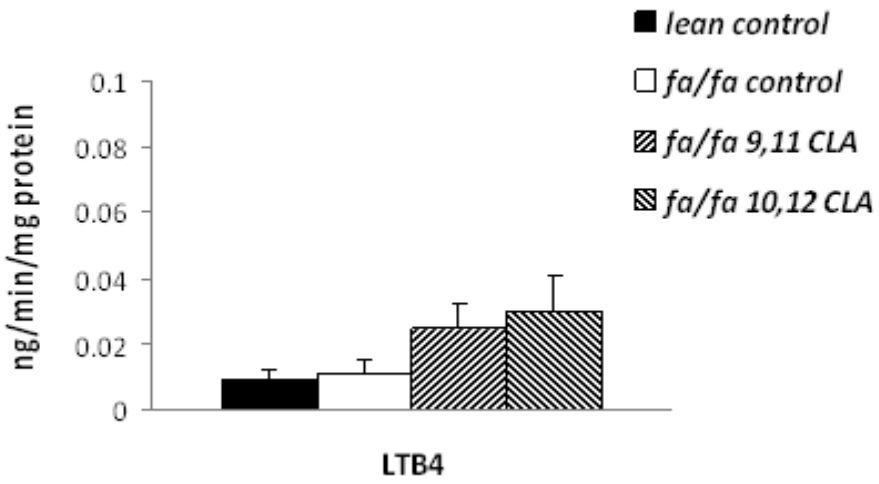
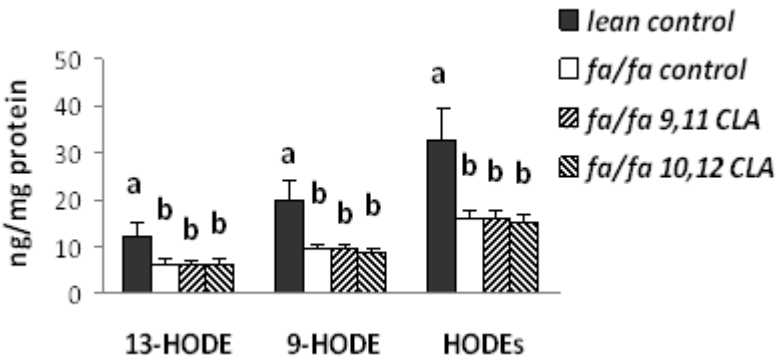


Figure 24. Liver LTB_4 . Endogenous levels (ng/mg protein) (A) and in vitro production (ng/min/mg protein) (B) of liver LTB_4 in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=7 in each group). Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other.

A



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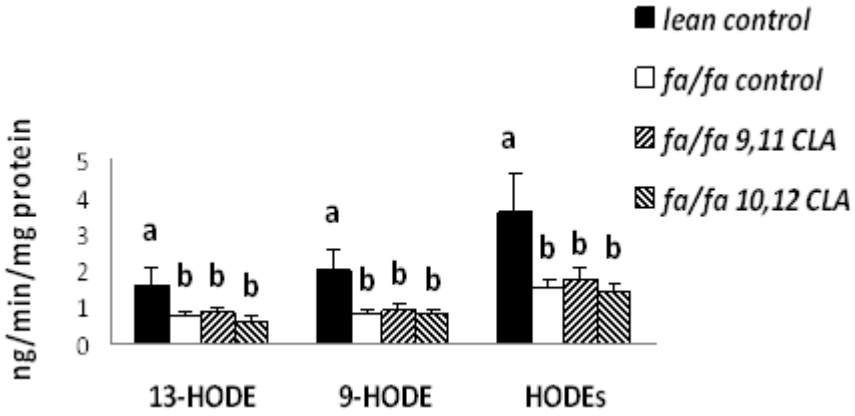


Figure 25. Liver HODEs. Endogenous levels (ng/mg protein) (A) and in vitro production (ng/min/mg protein) (B) of liver HODEs in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=7 in each group). Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other.

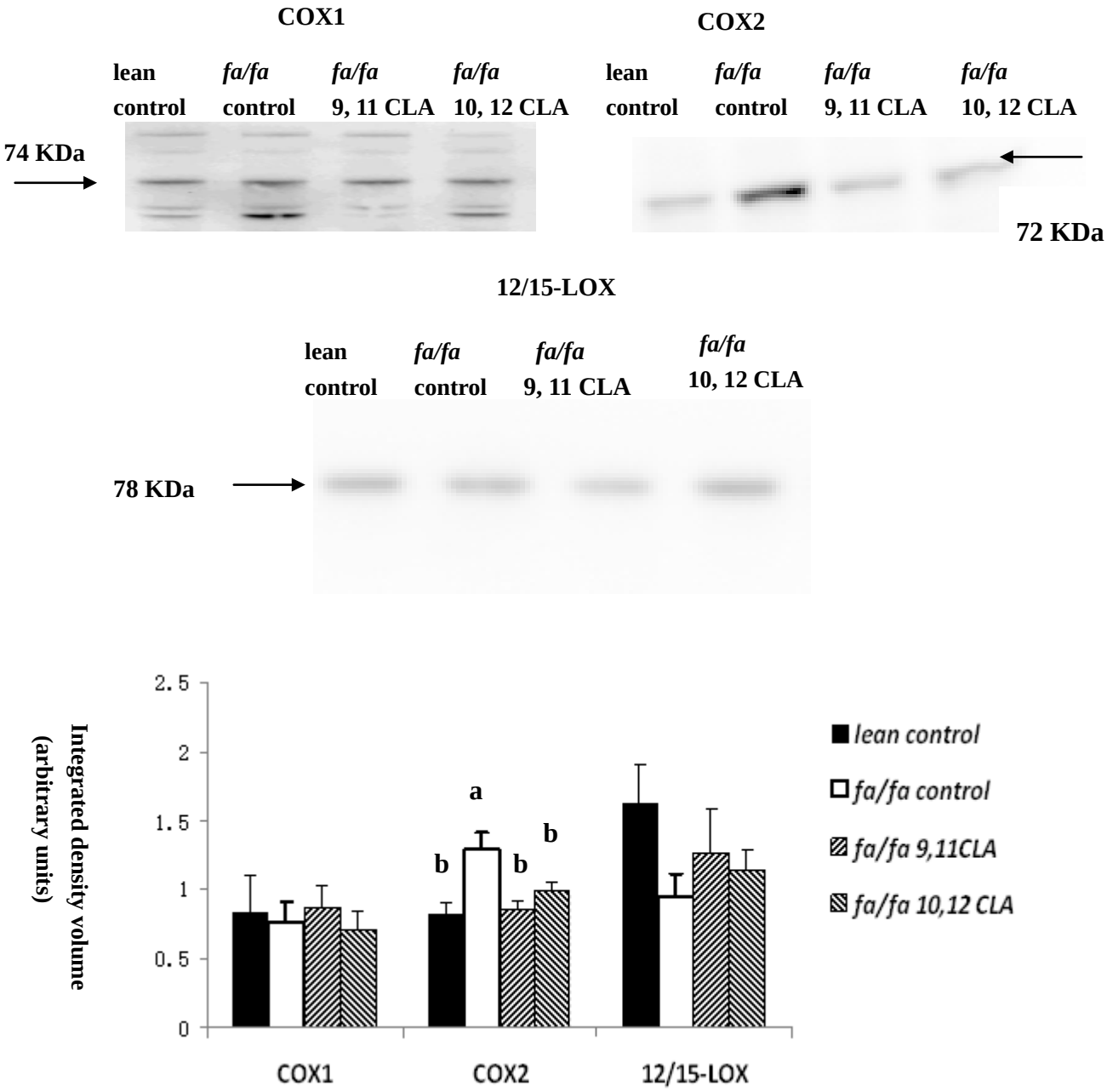


Figure 26. Liver COX1, COX2, and 12/15-LOX. Protein levels of COX1, COX2, and 12/15-LOX of liver in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=7 in each group). Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other. The upper panel shows representative blots.

significantly decreased the protein level of COX2. Although, the in vitro production of 13-HODE may indicate that lean Zucker rats had higher activity of 12/15-LOX than *fa/fa* rats, the protein levels of 12/15-LOX were not significantly different between these two groups and CLA treatment did not alter it. In terms of the enzymes involved in LTB_4 synthesis, 5-LOX located in either the cytoplasm or the nucleus translocates to membranes to initiate leukotriene synthesis from AA upon cell stimulation (Luo et al, 2003). We attempted to detect 5-LOX by Western blotting. However when up to 100 μg of protein was used, we could not detect any bands for 5-LOX using 1:200 and 1:100 dilutions, respectively, of 5-LOX antibodies (Cat. 160402, Cayman or Cat. Sc 8885, Santa Cruz).

3-3. Endogenous levels and in vitro production of eicosanoids in kidney

The endogenous levels and in vitro production of 14 eicosanoids detected in kidney are summarized in Table 12 and Table 13, respectively. As indicated in Figure 27, even though endogenous levels of 6-k-$PGF_{1\alpha}$ and TXB_2 were not significantly different between lean and *fa/fa* rats given control diet, *t*10,*c*12 CLA treatment resulted in higher 6-k-$PGF_{1\alpha}$ levels compared to *c*9,*t*11 CLA treatment ($p<0.05$), and higher TXB_2 levels compared to *c*9,*t*11 CLA ($p<0.005$) and *fa/fa* control groups ($p<0.05$). However, as shown in Figure 27, the differences in in vitro production of these prostanoids between groups were not significant.

Similar to the liver results, lean compared to *fa/fa* control rats had higher levels of renal 9-HODE ($p<0.001$), 13-HODE ($p<0.01$) and total HODE levels ($p<0.005$) (Figure

Table 12. Endogenous levels of kidney eicosanoids in lean and *fa/fa* rats given control diets and *fa/fa* rats given 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (ng/mg protein)

Analyte Name	lean control (n=6)	*fa/fa* control (n=6)	*fa/fa* 9,11 CLA (n=6)	*fa/fa* 10,12 CLA (n=6)
6-k-PGF$_{1\alpha}$	0.81 ± 0.11^{b}	1.30 ± 0.12^{ab}	1.14 ± 0.26^{b}	1.94 ± 0.40^{a}
TXB$_2$	0.49 ± 0.06^{b}	0.83 ± 0.13^{b}	0.59 ± 0.08^{b}	1.30 ± 0.24^{a}
PGF$_{2\alpha}$	1.17 ± 0.21	1.03 ± 0.10	1.11 ± 0.15	1.40 ± 0.22
PGE$_2$	2.43 ± 0.73	1.61 ± 0.27	2.20 ± 0.67	3.29 ± 1.28
PGD$_2$	0.60 ± 0.10	0.52 ± 0.09	0.51 ± 0.08	0.94 ± 0.19
PGs	5.49 ± 1.14	5.29 ± 0.62	5.55 ± 1.16	8.86 ± 2.11
LTB$_4$	0.09 ± 0.02	0.12 ± 0.03	0.11± 0.01	0.07 ± 0.02
9-HODE	49.62 ± 3.99^{a}	25.88 ± 2.07^{b}	23.73 ± 2.35^{b}	26.45 ± 6.94^{b}
13-HODE	42.79 ± 4.82^{a}	25.74 ± 1.84^{b}	23.35 ± 2.62^{b}	24.80 ± 5.89^{b}
HODEs	92.40 ± 8.65^{a}	51.62 ± 3.82^{b}	47.08 ± 4.91^{b}	51.25 ± 12.79^{b}
5-HETE	21.58 ± 1.80	26.58 ± 2.72	24.48 ± 1.76	22.94 ± 6.65
8-HETE	2.63 ± 0.32	2.79 ± 0.31	2.84 ± 0.18	3.30 ± 0.81
9-HETE	2.06 ± 0.19	2.16 ± 0.23	2.03 ± 0.15	2.68 ± 0.74
11-HETE	26.75 ± 2.38	27.26 ± 1.99	27.36 ± 1.60	32.35 ± 6.01
12-HETE	8.53 ± 0.7	6.93 ± 1.23	6.87 ± 0.93	7.34 ± 1.45
15-HETE	15.10 ± 1.37	15.20 ± 2.09	13.31 ± 1.30	15.67 ± 4.22
20-HETE	0.48 ± 0.05	0.89 ± 0.11	0.65 ± 0.08	0.62 ± 0.14
HETEs	76.72 ± 6.35	80.93 ± 7.52	76.88 ± 4.41	84.28 ± 19.60

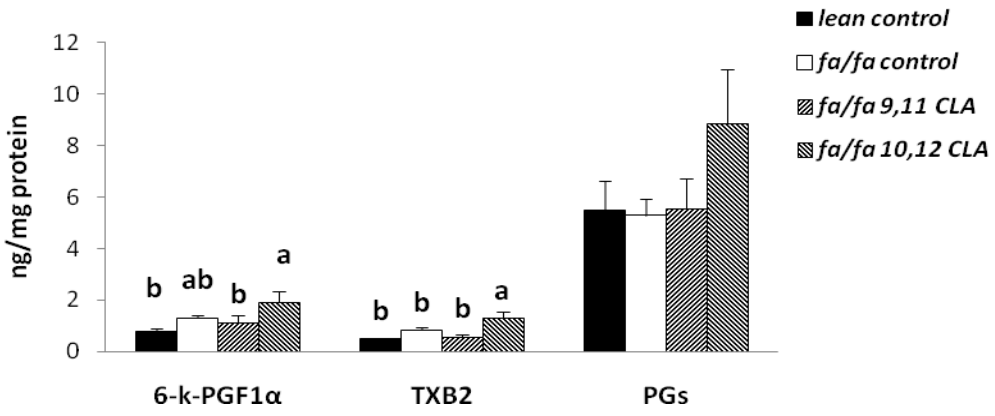
All data were expressed as mean ± SE. Values having different superscripts are significantly different from each other. P value <0.05 was considered statistically significant.

Table 13. In vitro production of kidney eicosanoids in lean and *fa/fa* rats given control diets and *fa/fa* rats given 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (ng/min/mg protein)

Analyte Name	lean control (n=6)	*fa/fa* control (n=6)	*fa/fa* 9,11 CLA (n=6)	*fa/fa* 10,12 CLA (n=6)
6-k-PGF$_{1\alpha}$	0.18 ± 0.03	0.25 ± 0.05	0.23 ±0.05	0.51 ± 0.19
TXB$_2$	0.10 ± 0.01	0.14 ± 0.02	0.15 ± 0.03	0.37 ± 0.14
PGF$_{2\alpha}$	0.55 ± 0.09	0.44 ± 0.05	0.51 ± 0.05	0.52 ± 0.07
PGE$_2$	0.27 ± 0.02	0.19 ± 0.03	0.22 ± 0.03	0.28 ± 0.07
PGD$_2$	0.29 ± 0.04	0.21 ± 0.04	0.16 ± 0.03	0.27 ± 0.06
PGs	1.38 ± 9.18	1.24 ± 0.12	1.29 ± 0.16	1.95 ± 0.48
LTB$_4$	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.002
9-HODE	6.01 ± 0.56^{a}	3.20 ± 0.46^{b}	3.32 ± 0.37^{b}	2.05 ± 0.52^{b}
13-HODE	5.51 ± 0.33^{a}	3.36 ± 0.18^{b}	3.06 ± 0.33^{b}	1.76 ± 0.47^{c}
HODEs	11.52 ± 0.88^{a}	6.57 ± 0.59^{b}	6.38 ± 0.67^{b}	3.80 ± 0.95^{c}
5-HETE	1.84 ± 0.19^{ab}	2.44 ± 0.36^{a}	2.53 ± 0.29^{a}	0.96 ± 0.52^{b}
8-HETE	0.37 ± 0.05^{a}	0.30 ± 0.04^{a}	0.27 ± 0.05^{a}	0.11 ± 0.06^{b}
9-HETE *	0.21 ± 0.02^{a}	0.21 ± 0.04^{a}	0.21 ± 0.03^{a}	0.08 ± 0.05^{b}
11-HETE	3.21 ± 0.38	3.39 ± 0.31	3.25 ± 0.22	2.65 ± 0.62
12-HETE	1.41 ± 0.07^{a}	0.80 ± 0.05^{bc}	0.87 ± 0.06^{b}	0.63 ± 0.07^{c}
15-HETE	1.76 ± 0.12^{a}	1.59 ± 0.11^{a}	1.58 ± 0.11^{a}	0.99 ± 0.20^{b}
20-HETE	0.03 ± 0.01	0.04 ± 0.05	0.05 ± 0.01	0.03 ± 0.03
HETEs	8.80 ± 0.66^{a}	8.73±0.70^{a}	8.72 ± 0.64^{a}	5.42 ± 1.33^{b}

All data were expressed as mean ± SE. Values having different superscripts are significantly different from each other. P value <0.05 was considered statistically significant. * P=0.0539

A



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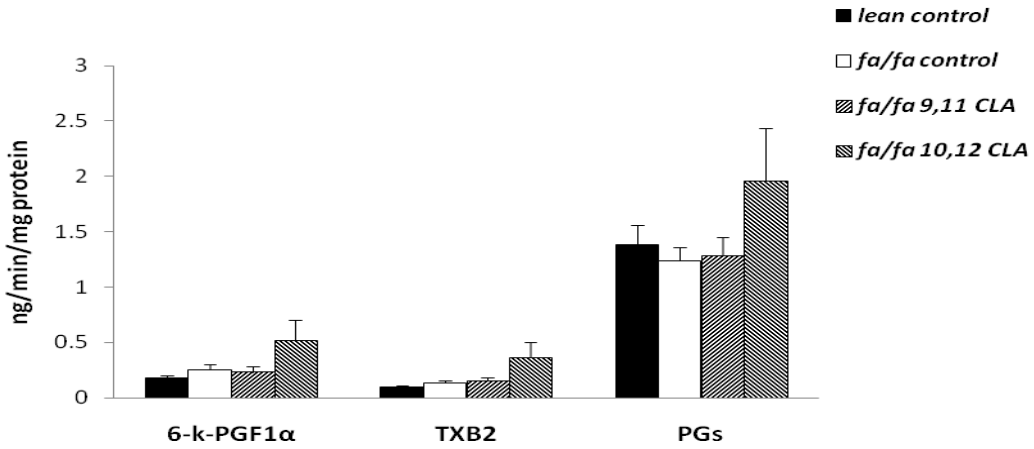

Figure 27. Kidney 6-keto-prostaglandin $F_{1\alpha}$ (6-k-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2). Endogenous levels (ng/mg protein) (A) and in vitro production (ng/min/mg protein) (B) of kidney 6-k-$PGF_{1\alpha}$ and TXB_2 in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=6 in each group). Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other.

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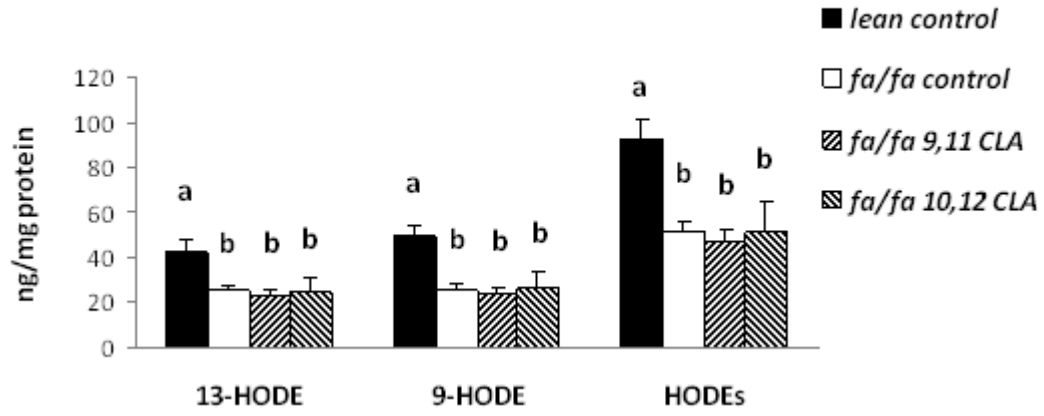

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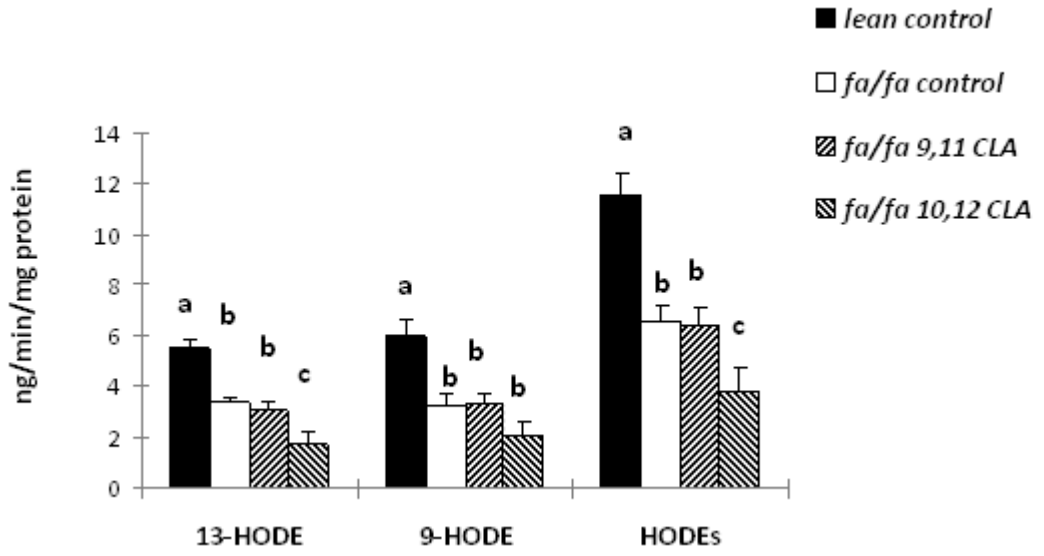


Figur 28. Kidney HODEs. Endogenous levels (ng/mg protein) (A) and in vitro production (ng/min/mg protein) (B) of kidney HODEs in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=6 in each group) . Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other.

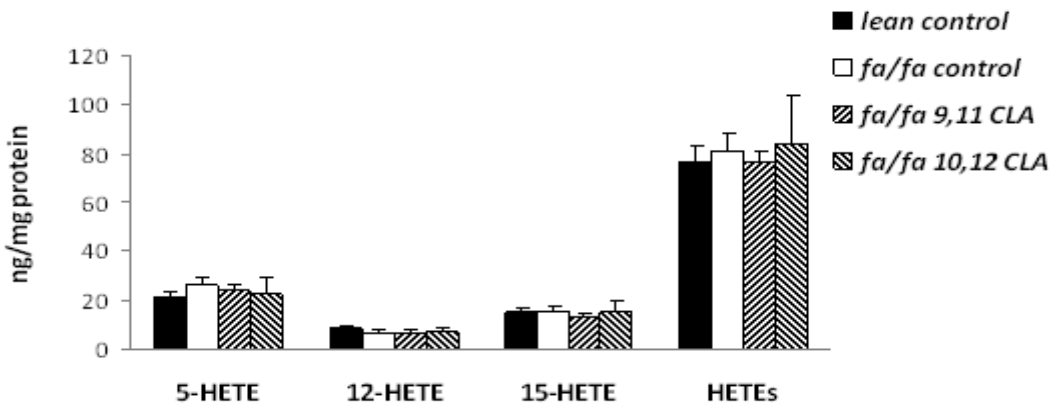
28) and higher in vitro production ($p<0.001$) of these two individual and total HODEs (Figure 28). Both CLA isomer treatments did not affect endogenous HODE levels; however, *t*10,*c*12 CLA significantly decreased the in vitro production of 13-HODE and total HODEs compared to *fa/fa* rats fed the control ($p<0.001$) or *c*9,*t*11 CLA diet ($p<0.05$).

In contrast to the HODEs, only the in vitro production of 12-HETE was significantly different in lean compared to *fa/fa* control rats. With respect to dietary treatment, *t*10,*c*12 CLA reduced the in vitro production of 15-HETE ($p<0.01$ vs. *fa/fa* group and *c*9,*t*11 CLA group), 5-HETE ($p<0.01$ vs. *fa/fa* group and *c*9,*t*11 CLA group), 8-HETE ($p<0.05$ vs. *fa/fa* group and *c*9,*t*11 CLA group) and total HETEs ($p<0.05$ vs. *fa/fa* group and *c*9,*t*11 CLA group) (Figure 29 and 30). In vitro production of 9-HETE followed a similar trend (overall P value = 0.054) (Figure 30), with the *t*10,*c*12 CLA group having lower 9-HETE production levels than the other groups. In vitro production of 12-HETE in the *t*10, *c*12 CLA compared to *c*9,*t*11 CLA also was lower ($p<0.001$) (Figure 27). When the production of 13-HODE and 12-HETE were combined (Figure 31), *fa/fa* compared to lean rats had lower levels of these products and in vitro production of the 12/15-LOX enzyme, and rats given *t*10,*c*12 CLA had even lower in vitro production, but *t*10,*c*12 CLA did not affect the endogenous levels of these two production.

3-4. Protein levels of enzymes involved in eicosanoid metabolism in kidney

Since some prostanoids were different between groups, COX1 and COX2 were examined by Western blotting. Renal COX1 was not significantly different between genotypes; however, *t*10,*c*12 CLA significantly increased its protein levels ($p<0.05$ vs. *fa/fa* control group) (Figure. 32). Further correlation analysis between COX1 and TXB_2

A



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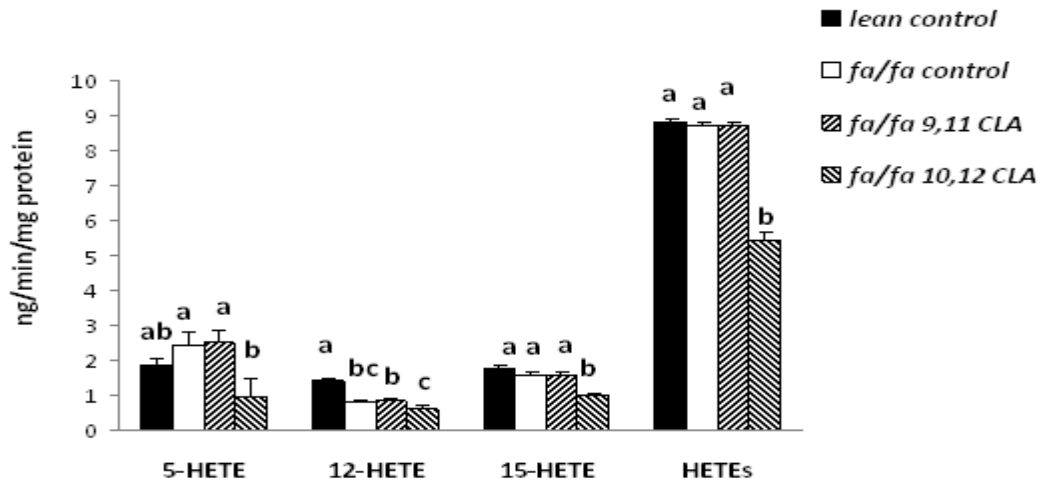

Figure 29. Kidney HETEs. Endogenous levels (ng/mg prot n) (A) and in vitro production (ng/min/mg protein) (B) of kidney HETEs in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=6 in each group) . Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other.

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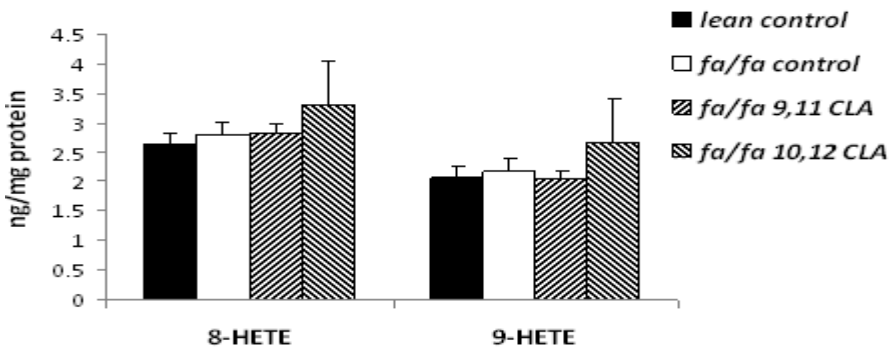

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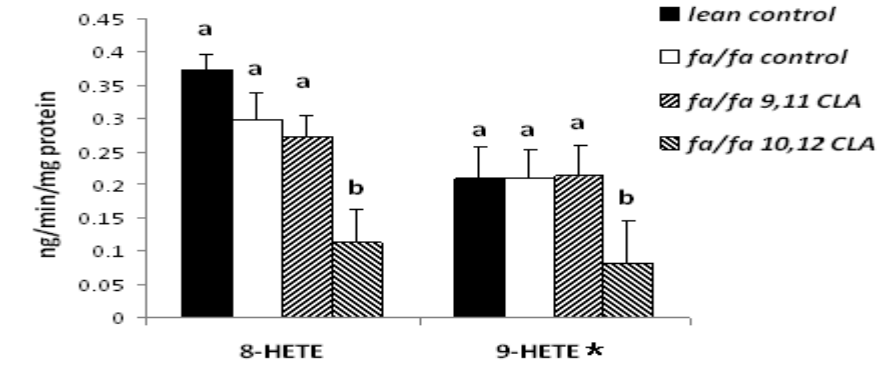
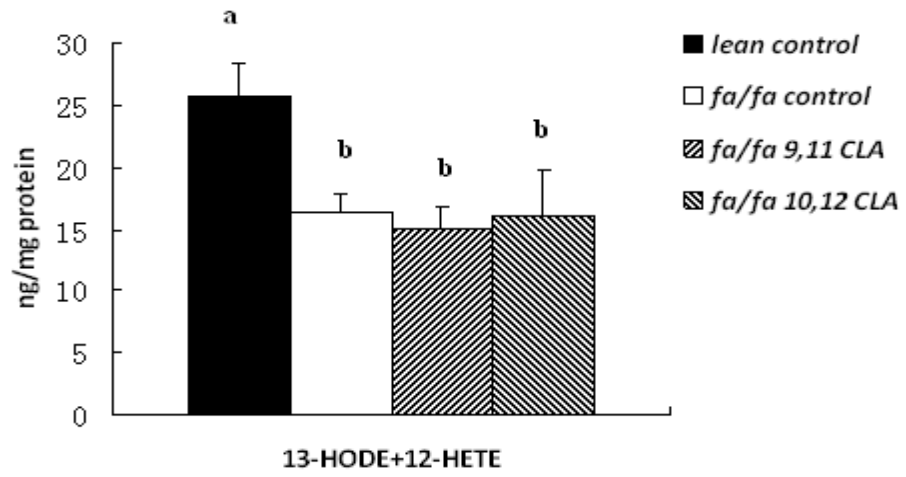


Figure 30. Kidney 8- and 9-HETE. Endogenous levels (ng/mg protein) (A) and in vitro production (ng/min/mg protein) (B) of kidney 8-, and 9-HETE in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=6 in each group). Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other. * P =0.05 for ANOVA test

A



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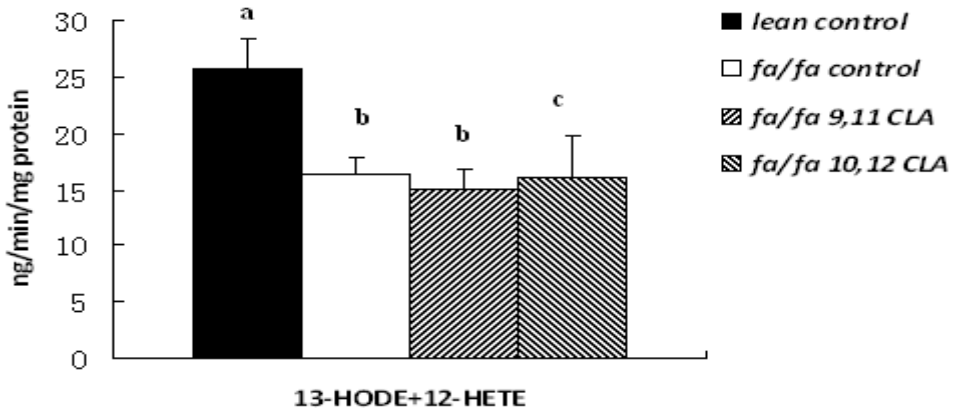


Figure 31. Total 13-HODE and 12-HETE in kidney. Endogenous levels (ng/mg protein) (A) and in vitro production (ng/min /mg protein) (B) of total of 13-HODE and 12-HETE in kidney in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA(n=6 in each group) . Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other.

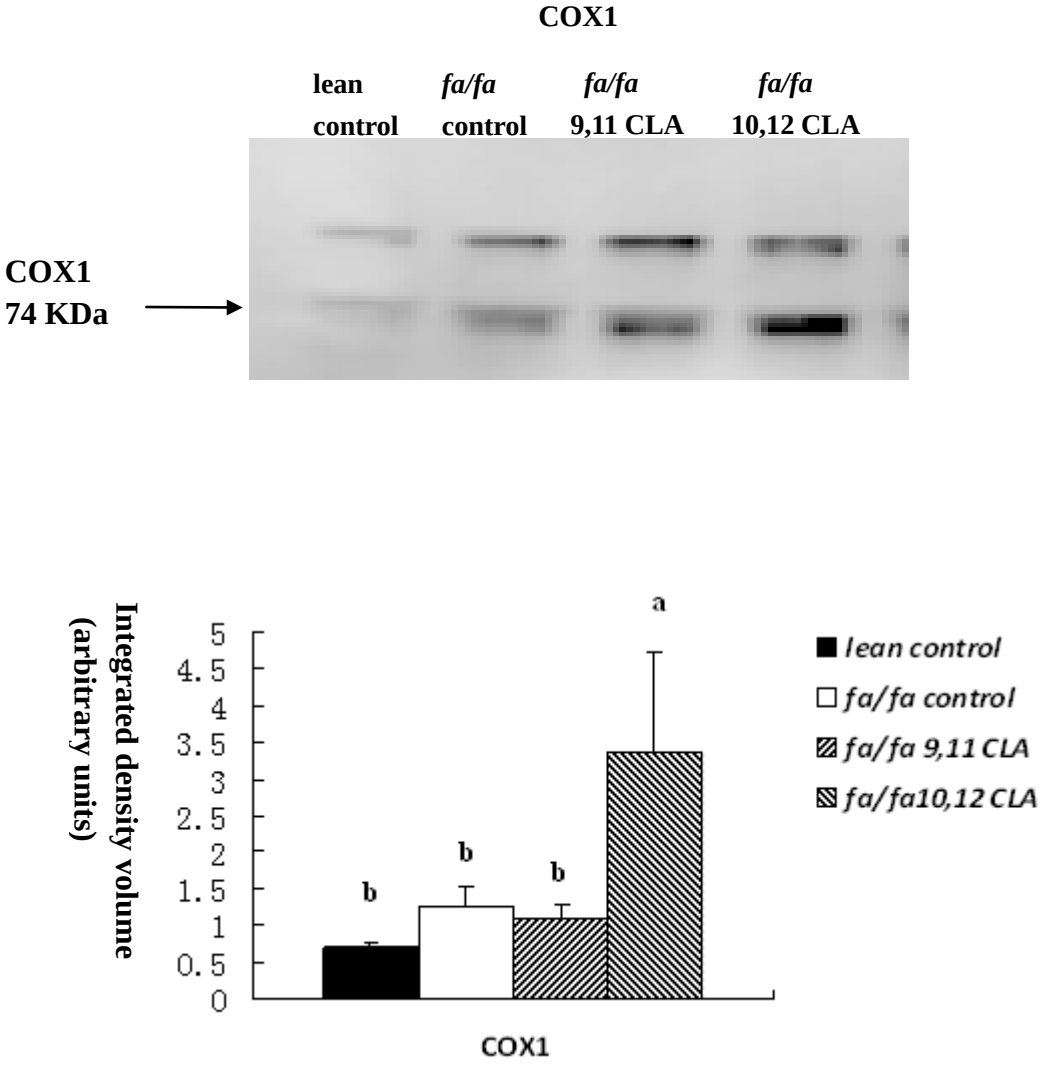


Figure 32. Kidney COX1. Protein levels of COX1 of kidney in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=6 in each group). Values are expressed as mean ± SE. Values having different superscripts are significantly different different from each other. The upper panel shows the representative blots.

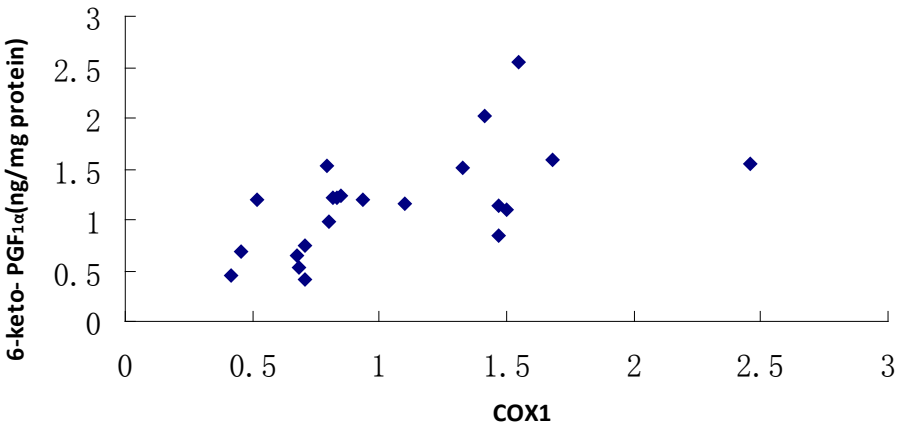


Figure 33. Correlation of COX1 and endogenous levels of 6-keto-$PGF_{1\alpha}$ in kidney.

COX1 was positively correlated with endogenous levels of 6-keto-$PGF_{1\alpha}$ in kidney (r = 0.66, p = 0.00083). Two outliers have been removed from this analysis. When the outliers are included, however, the r value is higher and the significance is greater (see Appendix Figure 1 for data including outliers).

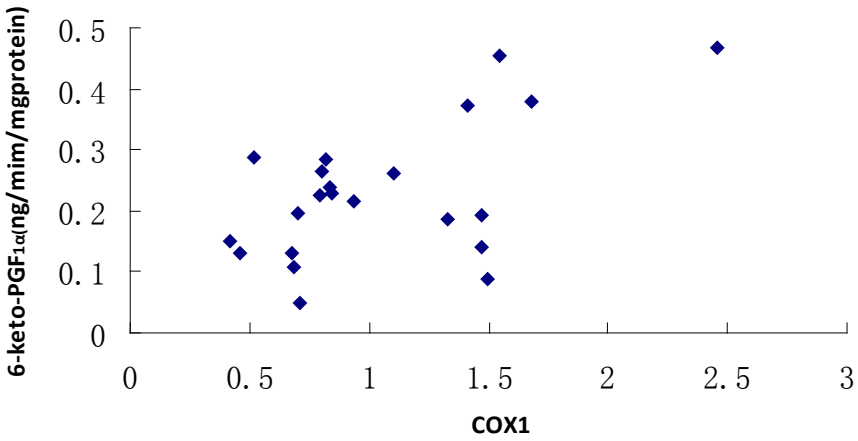


Figure 34. Correlation of COX1 and in vitro production of 6-keto-PGF_{1a} in kidney.

COX1 was positively correlated with the in vitro production of 6-keto-$PGF_{1\alpha}$ ($r = 0.43$, $p = 0.0443$). Two outliers have been removed from this analysis. When the outliers are included, however, the r value is higher and the significance is greater (see Appendix Figure 2 for data including outliers).

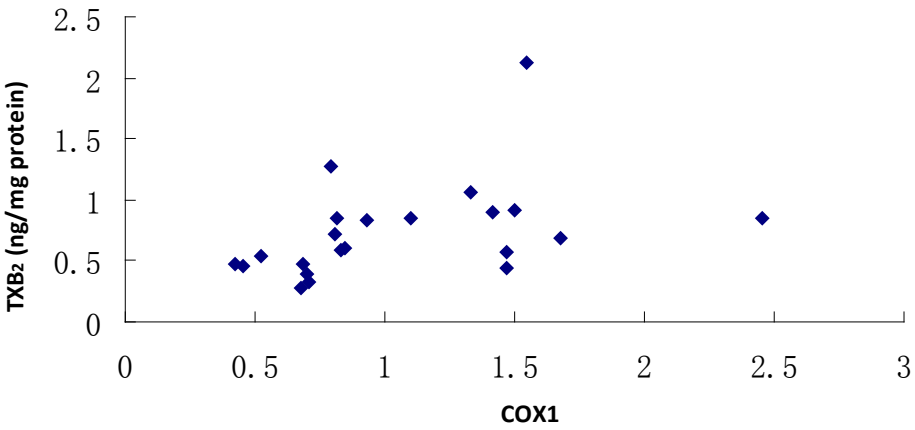


Figure 35. Correlation of COX1 and endogenous levels of TXB_2 in kidney. COX1 was positively correlated with the endogenous levels of TXB_2 ($r = 0.58$, $p = 0.0045$). Two outliers have been removed from this analysis. When the outliers are included, however, the r value is higher and the significance is greater (see Appendix Figure 3 for data including outliers).

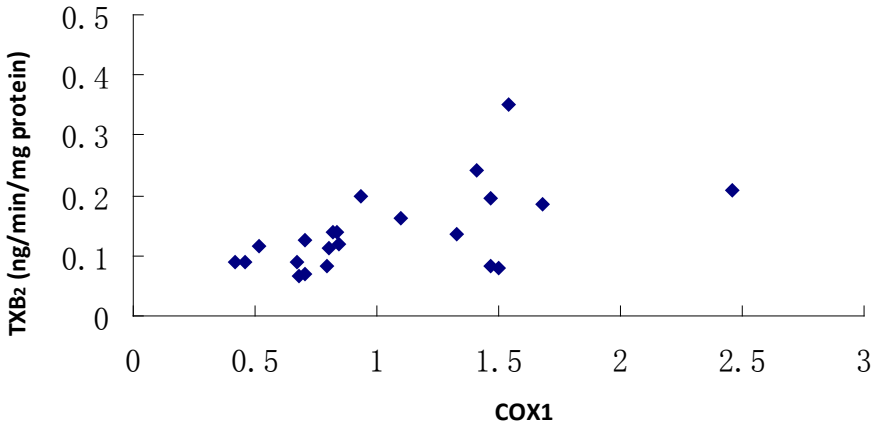
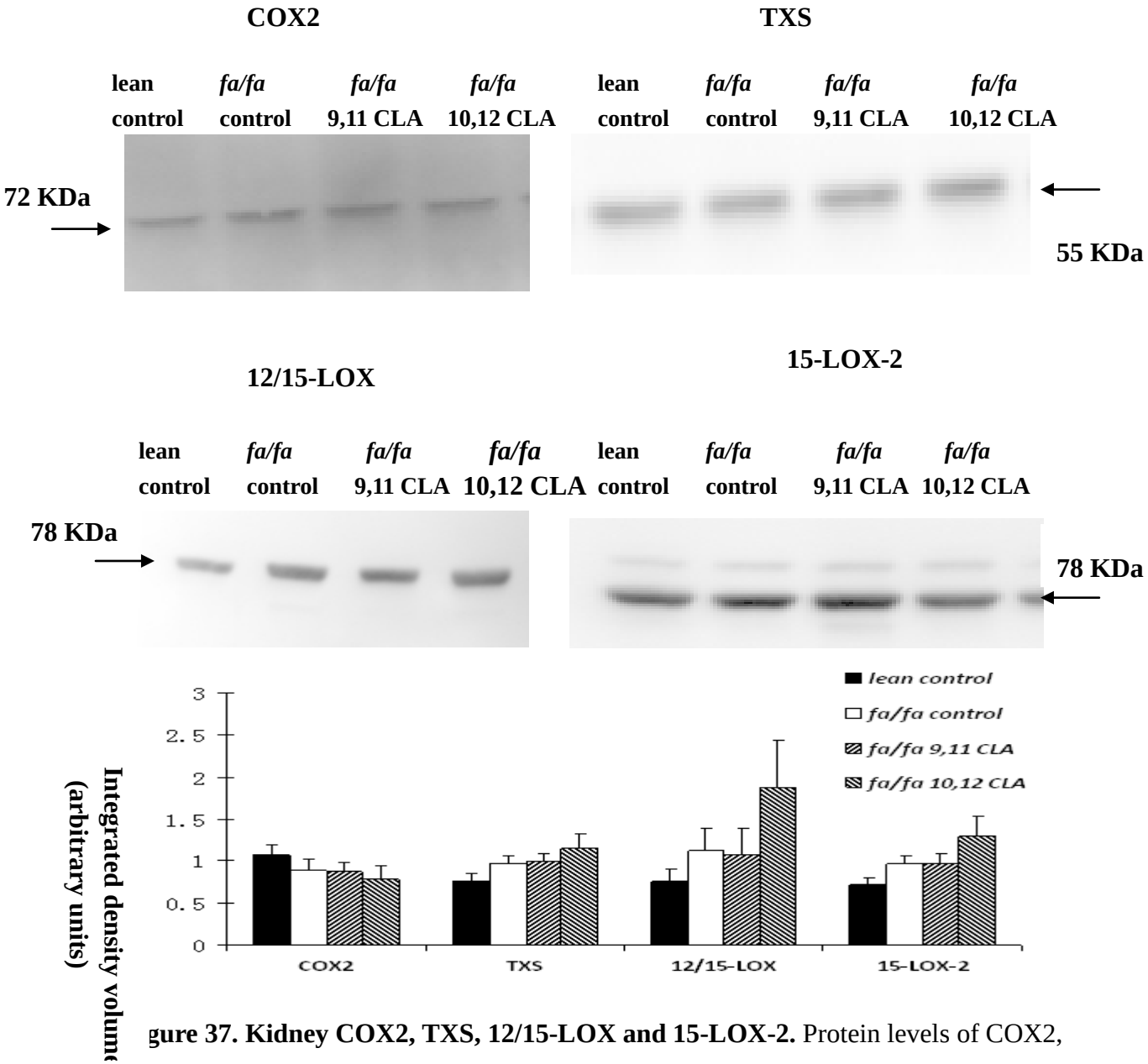


Figure 36. Correlation of COX1 and in vitro production of TXB_2 in kidney. COX1 was positively correlated with the in vitro production of TXB_2 ($r = 0.58$, $p = 0.0049$). Two outliers have been removed from this analysis.When the outliers are included, however, the r value is higher and the significance is greater (see Appendix Figure 4 for data including outliers).



gure 37. Kidney COX2, TXS, 12/15-LOX and 15-LOX-2. Protein levels of COX2, TXS, 12/15-LOX and 15-LOX-2 of kidney in lean or *fa/fa* rats given control diets (no CLA) and *fa/fa* rats given control diet plus 0.4% (w/w) *c*9,*t*11 CLA or 0.4% (w/w) *t*10,*c*12 CLA (n=6 in each group). Values are expressed as mean ± SE. Values having different superscripts are significantly different from each other. The upper panel shows the representative blots.

and PGI_2 with (Appendix-Figure1-4) or without (Figures 33-36) two outliers showed that COX1 positively correlated with the endogenous levels and in vitro production of TXB_2 and PGI_2, but not any of the other prostanoids (not shown). Protein levels of COX2 and TXS were not altered (Figure. 37) by genotype or diet. To detect enzymes possibly related to HETE and HODE synthesis, 12/15-LOX, 15-LOX-2, and 5-LOX were examined by Western blotting. Neither genotype nor CLA isomers affected the levels of these proteins (Figure. 37). With respect to 8-LOX and 9-LOX, commercial antibodies for the detection of these proteins in Western blotting are not available. We attempted to detect 5-LOX (Cat. 160402, Cayman and sc-8885, Santa Cruz) and PGIS (Cat. 100023, Cayman) in kidney, using up to 100 μg and 60 μg of protein, respectively, but we were unable to detect bands for either of these two enzymes.

4. DISCUSSION

Inflammation is linked with many chronic diseases. Different eicosanoids as well as cytokines are involved in inflammation as chemical mediators to promote or attenuate inflammation. CLA exerts anti-inflammatory effects through alteration of body composition, interference with enzymes related to the formation of eicosanoids, PPAR-γ and NF-κB (Banni, 2002; Stachowska et al, 2007a; Ochoa et al, 2004; Kim et al, 2005; Stachowska et al, 2007b; Cho et al, 2005; Yu et al, 2002; and Varga and Nagy, 2008). Because of the structural differences in CLA isomers, including *c*9,*t*11 CLA and *t*10,*c*12 CLA, they play different roles in lipid metabolism, incorporation into tissues, and in the effect on PPAR-γ and NF-κB. Furthermore, it has been documented that CLA effects are species-, tissue-, as well as isomer-specific (Tsuboyama et al, 2000; Wargent et al, 2005 and Feitoza et al, 2009). Currently, few in vivo studies have investigated the effect of CLA isomers on multiple eicosanoids in tissues of obese animal models.

4-1. Liver discussion

Several studies have indicated that COX1, COX2, PGI_2, TXB_2 (Cao et al, 2008) and 5-LOX, 12-LOX and their metabolites (López-Parra et al, 2008 and Martínez-Clemente et al, 2010) are implicated in liver damage associated with obesity. The present study found that even though PGs were not altered in liver of *fa/fa* rats, higher COX2 levels were present in livers of *fa/fa* rats compared to lean Zucker rats. NF-κB is known to regulate the transcription of COX2, as well as inflammatory molecules such as TNF-α. One of the mechanisms by which NF-κB is activated is oxidative stress resulting from the uncontrolled production of ROS. During the conversion of PGG_2 to PGH_2 by COX, ROS

can be generated which can activate NF-κB, forming an auto activating loop during inflammation (Chung et al, 2009). Cao et al further ascertained this role of COX2 in a liver steatosis model of high fat-induced obese rats (Cao et al, 2008) in which increments in COX2 were associated with liver injury.

CLA down-regulates COX2 protein expression through activation of PPAR-γ and PPAR-γ-mediated inhibition of NF-κB (Yu et al, 2002 and Varga and Nagy, 2008). In accordance with the study of Moloney (Moloney et al, 2007), which confirmed that *c*9,*t*11 CLA exerted an anti-inflammatory effect through down-regulation of NF-κB p65 expression, NF-κB DNA binding, and NF-κB p65, p50, c-Rel, p52, and RelB transcriptional activity in adipose tissue, the present study found that *c*9,*t*11 CLA decreased COX2 protein levels. On the other hand, Hommelberg et al (Hommelberg et al, 2010) recently demonstrated that *t*10,*c*12 CLA increased the levels of inflammatory biomarkers through increased NF-κB RelA DNA binding and would be expected to also increase COX2 in muscle cells. However, *t*10,*c*12 CLA decreased the protein levels of COX2 in the present study. This reduction may be due to the lower total fat content of liver in the *t*10,*c*12 CLA fed compared to *fa/fa* control *rats* (unpublished data, C. Taylor et al), or may have been due to the activation of PPAR-γ (Varga and Nagy, 2008).

Further evidence of increased inflammation in the *t*10,*c*12 CLA group is implied by the elevated LTB_4 levels observed in livers in this group. 5-LOX is primarily expressed in macrophages (Khanapure et al, 2007) and LTB_4 is considered to be a potent chemotactic mediator for the infiltration and activation of neutrophils in inflammation, leading to tissue damage (Wang et al, 2000). As well, López-Parra et al (López-Parra et al, 2008) found that

5-HETE and LTB_4 were elevated in hepatic steatosis in obese mice, which was not the case for either endogenous levels or in vitro production of these eicosanoids in the current study with *fa/fa* compared to lean rats. In contrast to the finding of Sugano et al (Sugano et al, 1998), who found that a mixture of CLA isomers decreases PGE_2 and LTB_4 in peritoneal exudate cells, spleen and lung, *t*10,*c*12 CLA increased liver LTB_4 level in *fa/fa* rats in the current study. Histological analysis including the infiltration of macrophages and generation of fibrosis may help elucidate whether the putative beneficial effect of reduced COX2 is counterbalanced by the elevated liver LTB_4 observed in the *t*10,*c*12 CLA fed rats. Further, Western blotting analysis of 5-LOX, FLAP and LTA_4 hydrolase enzymes may shed light on the mechanism underlying this increase in LTB_4 in the *t*10,*c*12 CLA group because all of these 3 factors are involved in the generation of LTB_4 .

The present study showed that lean compared to *fa/fa* rats had higher levels of 13-HODE and 9-HODE. Previous analysis of the fatty acid composition of this liver tissue (unpublished data, C Taylor et al) showed that lean compared to *fa/fa* rats had higher hepatic LA levels. This observation, along with the absence of differences in the protein levels of COX1 and 12/15-LOX in lean compared to *fa/fa* rats, suggests that the differences in HODE content could be due to substrate differences. It is also possible that post-transcriptional (non-enzymatic) factors play a role in the differing HODE levels. The fact that COX2 levels are not consistent with HODE levels suggests that this isoform is not critical to HODE formation in the liver. In terms of the mechanism by which lean compared to *fa/fa* rats have higher LA levels, it may be due to alteration in desaturase enzyme activities. It has been reported that Δ9 desaturae activity is elevated and Δ5 and Δ6

desaturases are reduced in liver steatosis (Allard et al, 2008).

Feldstein et al demonstrated (Feldstein et al, 2010) that the patients with nonalcoholic fatty liver compared to control subjects had higher levels of circulating 13-HODE and 9-HODE, reflecting high oxidative stress. This finding is opposite to the liver results in the present study. Possible explanations for this discrepency may be due to species differences, or the source of HODEs could be different between liver tissue and systemic blood: 1) the HODEs in the circulating system are from oxidation of LDL (Negishi et al, 2004). 2) the HODEs in liver tissue in the current study presumably are derived from LA in tissue lipids.

4-2. Kidney discussion

Previous histological analysis of kidney from the rats analyzed in the current study revealed that *t*10,*c*12 CLA treatment decreased glomerular volume, worsened glomerular damage and increased macrophage infiltration (unpublished data, Y Zhen). In the current eicosanoid analysis, endogenous levels of select prostanoids and COX1 protein levels were elevated and the in vitro production of HETEs was reduced by *t*10,*c*12 CLA feeding.

As discussed above, several studies have reported controversial results regarding the protein levels of COX1 and COX2 in obesity-induced kidney damage. For instance, Drury et al (2009) found that renal COX1 protein levels did not differ between 14 wk old male lean and *fa/fa* rats. However, COX2 protein levels was increased in *fa/fa* rats at this age, and 0.4% (w/w) *t*10,*c*12 CLA decreased renal COX2 protein levels. Another study undertaken by Komers et al (2005) also reported that increased protein levels of COX2 were present in renal cortex in 12 wk old male obese *fa/fa* rats and was accompanied by increased TXB_2 excretion in urine. However, COX1 protein levels were decreased. As

opposed to these studies, in the present study of 25 wk old male rats, neither COX1 nor COX2 levels were increased in kidneys of *fa/fa* compared to lean rats. Interestingly, *t*10,*c*12 CLA treatment increased COX1 protein levels accompanied by an increment in TXB_2 compared to *fa/fa* control rats, and increased PGI_2 levels compared to *c*9,*t*11 CLA treatment. However, COX2 was not altered by CLA treatment. Correlation analysis between COX1 and prostanoids showed that COX1 positively correlated with the endogenous levels and in vitro production of PGI_2 and TXB_2, but not any other prostanoids.

As pointed out above, PGI_2 is a potent vasodilator, an inhibitor of leukocyte adhesion and platelet aggregation and inhibitor of smooth muscle cell proliferation (Harada et al, 1999). In contrast, TXA_2 causes platelet aggregation, vasoconstriction, and vascular proliferation (Cipollone et al, 2008). Similarly, in kidney, PGI_2 and TXA_2 have opposite effects on the glomerular matrix (Bruggema et al, 1993). TXA_2 induces mRNAs of collagen IV, laminin, and fibronectin, increases extracellular matrix expansion, and causes glomerulosclerosis, but suppresses synthesis of heparan sulfate proteoglycan; however, PGI_2 has opposite effects to counteract these effects of TXA_2. Okumura et al demonstrated this alteration of PGI_2 and TXA_2 in glomerulosclerosis in a diabetic rat model (Okumura et al, 2000). They found that albuminuria increased with an increase in TXA_2 and PGI_2 synthesis. In accordance with the study of Okumura, Aparajita et al (2004) also found increased urine excretion of TXB_2 and 6-keto$PGF_{1\alpha}$ as well as decreased PGE_2 in *fa/fa* Zucker rats at 20–21wk of age. Consequently, in the current study, the higher TXB_2 levels caused by dietary *t*10,*c*12 CLA may contribute to the renal histological damage in *fa/fa* rats.

The elevated PGI_2 in *fa/fa* rats given *t*10,*c*12 CLA compared to lean rats in the current study may be a compensatory response to the increased TXB_2 level.

In human mesangial cells (Negishi et al, 2004), 9-HODE and 13-HODE promote proliferation and increase the production of both Type IV collagen and fibronectin in the extracellular matrix. Hence, the lower renal HODE levels in *fa/fa* compared to lean rats may be expected to be protective. This also may be a reflection of the different LA levels between genotypes. Guesnet et al (Guesnet et al, 1990) reported that kidney has similar fatty acid composition to liver which in *fa/fa* rats is characterized by impaired synthesis of polyunsaturated fatty acid and increased monounsaturated fatty acid synthesis by Δ9 desaturase. Considering the positive role of 13-HODE and 9-HODE in the vascular system (Fang et al, 1999 and Shen et al, 1996), the high levels of HODEs in lean rats might play a protective role in preventing atherosclerosis in the kidney.

In contrast to the up-regulation of 12/15-LOX expression and its metabolites in kidney damage of *fa/fa* rats in Xu's study (Xu et al, 2005), in the current study, 12/15-LOX activity as estimated by production of 12-HETE and 13-HODE was decreased in *fa/fa* rats compared to lean rats. This suggests that 12/15-LOX and its metabolites may confer a renal protective effect in lean rats. Moreover, our current study found that *t*10,*c*12 CLA decreased the activity of 12/15-LOX as estimated by production of 13-HODE and 12-HETE in kidney of *fa/fa* rats. In this regard, the inhibition of 12/15-LOX activity in kidney by *t*10,*c*12 CLA treatment might contribute to the deleterious effect of *t*10,*c*12 CLA in kidneys of *fa/fa* rats. In contrast, *t*10,*c*12 CLA treatment did not affect the enzyme activity resulting in the formation of 9-HODE. This may be due to the finding that

9(R)-HODE appears to be produced by COX activity as the major enzymatic product of LA along with 13(S)-HODE (Camacho et al, 1995).

The reduced enzyme activities for the synthesis of HETEs in *t*10,*c*12 CLA fed rats along with the worsened renal pathology suggests that these enzymes and their metabolites are also protective to the kidney. This is at odds with the traditional perception of HETEs as pro-inflammatory in the literature. For example, as discussed above, it has long been believed that 5-LOX and its metabolites mediate inflammatory reactions and glomerular immune injury as a potent chemoattractant (Hao and Breyer, 2007) and associate with the increased glomerular cell proliferation (Wu and Lianos, 1993). 12/15-LOX and its metabolite 12-HETE, which can be induced by high glucose and ANG II, are implicated in prediabetic kidney damage in obese *fa/fa* rats (Xu et al, 2005). Additionally, because increased LOX activity is a rich source of ROS generated during oxygenation and oxidative degradation of polyunsaturated fatty acids, many pathological effects of LOXs are accomplished through these ROS; hence, it is noted that not only 5-LOX and its metabolites, but also ROS generated from 5-LOX (Bonizzi et al, 1999) contribute to the onset of inflammation through NF-κB activation. Another study in mouse keratinocyte culture (Schweiger et al, 2007) confirmed it is the ROS rather than 8-HETE and 15-HETE generated from 8-LOX and 15-LOX-2 that inhibit cell growth in premalignant epithelial cells in carcinogenesis. However, if decreased activities of LOXs results in decreased production of their metabolites, such as lipoxins, which are anti-inflammatory, this may explain the detrimental effect of *t*10,*c*12 CLA on renal pathology in these rats. For instance, the lipoxins [lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4)] originated from 15-HETE

catalyzed by 5-LOX and LTA_4 catalyzed by 12-LOX (Fierro et al, 2000). Furthermore, a recent study found that 12/15-LOX and its metabolites are anti-atherosclerotic by promoting the efflux of cholesterol (Wittwer and Hersberger, 2007). Another study reported that 15-LOX-2 can activate PPAR-γ via protein–protein interaction between them (Fores et al, 2005). Subsequently, this activation of PPAR-γ can in turn inhibit NF-κB and the subsequent inflammatory events (Varga and Nagy, 2008).

5. CONCLUSION

Previous investigations in these liver and kidney tissues found that dietary *t*10,*c*12 CLA decreased the total fat content of liver but worsened renal damage (unpublished data, C Taylor et al and Y Zhen et al). The apparent positive effect of *t*10,*c*12 CLA on liver fat content coincides with increased LTB_4. Histological analysis of the liver will be needed in order to determine whether this effect indeed is protective and further studies examing changes in eicosanid biosynthetic enzymes are needed to further understand the mechanisms of this effect.

The detrimental effect of *t*10,*c*12 CLA on the kidney as evidenced by histological analysis correlates with increased levels of TXB_2, 6-keto-$PGF_{2\alpha}$ and the COX1 enzyme. Furthermore, reduced production of several HETEs and HODEs also are associated with the increased renal damage in the *t*10,*c*12 CLA fed group. Whether this altered eicosanoid profile is a cause or an effect of the renal alterations induced by dietary *t*10,*c*12 CLA remains to be elucidated.

6. LIMITATIONS

- 5-LOX and PGIS could not be detected in Western blotting possibly because this protein is highly expressed in macrophage and vascular endothelial cells, respectively, rather than in liver tissue cells.
- The fatty acid composition of kidney was not analyzed to determine whether the lean compared to *fa/fa* rat has higher levels of LA, which may lead to the higher 13-HODE and 9-HODE observed in lean rats.
- Storage of tissues at -80℃ may cause degredation of eicosanoids; however, there is no evidence for this. Tests in our lab (unpublished data) have demonstrated that eicosanoids are stable for more than 10 years when samples are stored at -80℃.

7. FUTURE RESEARCH

- The previous study showed that insulin resistance in *fa/fa* Zucker rats was improved by *t*10,*c*12 CLA . It will be interesting to investigate the alteration of eicosanoids by CLA in adipose and muscle tissue.
- Further detection of the mRNA levels of relevant enzymes in eicosanoid metabolism with real-time PCR will be helpful to determine whether CLA supplementation affects the expression of these enzymes at mRNA level.
- Because CYPs and EETs participate in drug metabolism in liver and hemodynamic regulation in kidney, the determination of CYPs and EETs in liver and kidney will be also novel in this area.
- Detection of the protein levels of 5-LOX with macrophage culture and the protein levels of LTA_4 hydrolase and FLAP with Western blotting will be helpful to understand the mechanisms by which *t*10,*c*12 CLA decreases the in vitro production of 5-HETE in kidney.
- Detection the protein levels of PGIS with vessel endothelial cell culture using Western blotting will be helpful to determine how *t*10,*c*12 CLA affects the protein expression of this enzyme in kidney.

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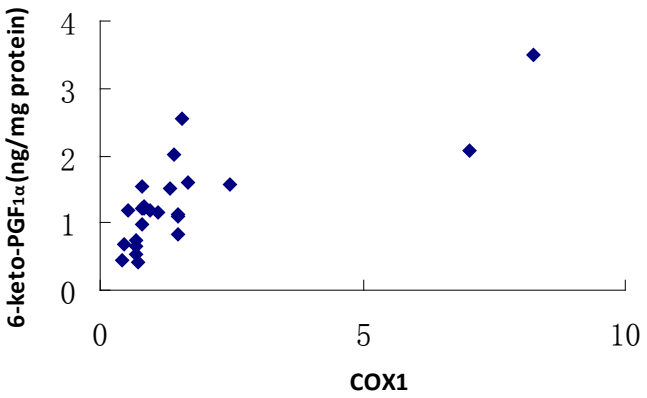
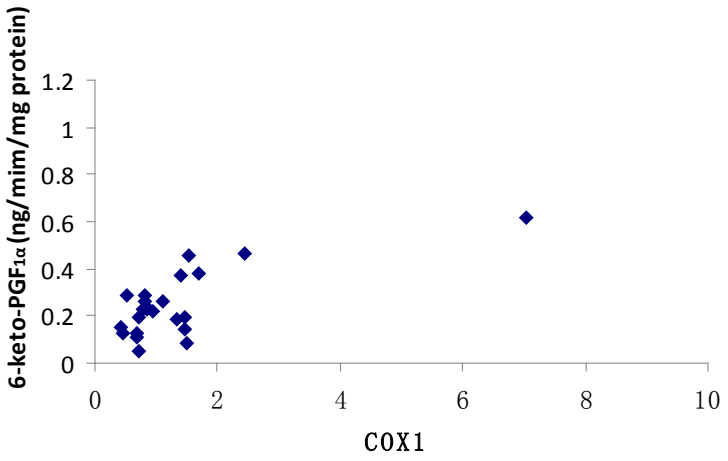
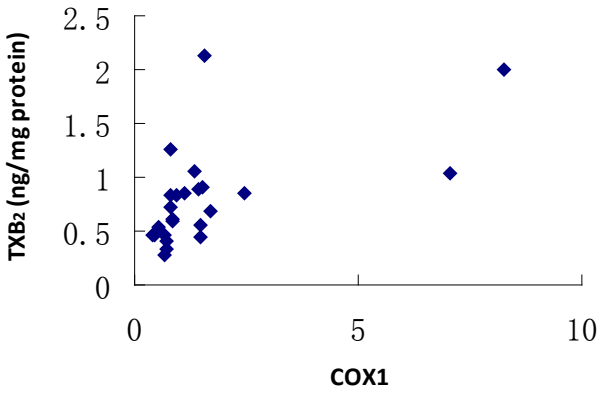
9. APPENDIX



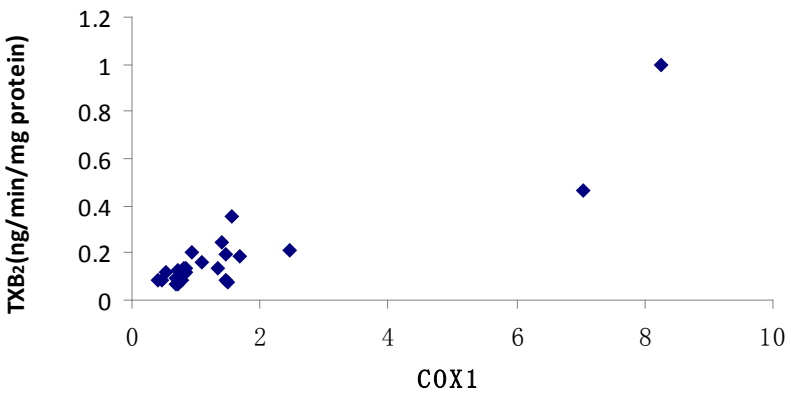
Appendix Figure 1. Correlation of COX1 and endogenous levels of 6-keto-$PGF_{1\alpha}$ in kidney. All data are included in this analysis. COX1 was positively correlated with endogenous levels of 6-keto-$PGF_{1\alpha}$ in kidney ($r = 0.73$, $p < 0.0001$).



Appendix Figure 2. Correlation of COX1 and in vitro production of 6-keto-$PGF_{1\alpha}$ in kidney. All data are included in this analysis. COX1 was positively correlated with in vitro production of 6-keto-$PGF_{1\alpha}$ in kidney ($r = 0.56$, $p = 0.0042$**)**.



Appendix Figure 3. Correlation of COX1 and endogenous levels of TXB_2 in kidney. All data are included in this analysis. COX1 was positively correlated with endogenous levels of TXB_2 in kidney ($r = 0.65$, $p = 0.0006$**)**.



Appendix Figure 4. Correlation of COX1 and in vitro production of TXB_2 in kidney. All data are included in this analysis. COX1 was positively correlated with in vitro production of TXB_2 in kidney ($r = 0.67$, $p = 0.0003$**)**.