INVESTIGATING THE THERAPEUTIC POTENTIAL OF DIETARY CONJUGATED LINOLEIC ACID (CLA) AND TELMISARTAN CO-ADMNISTRATION IN METABOLIC SYNDROME IN THE SPRAGUE-DAWLEY RAT

by

Mohammad M. Abdullah

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

Department of Human Nutritional Sciences

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ABSTRACT

Metabolic syndrome (MetS) is a term linking the clinical profiles of some of the 21st Century major health problems – obesity, diabetes, and heart disease. Both dietary conjugated linoleic acid (CLA) and the angiotensin II receptor blocker (ARB) telmisartan enhance features of MetS; however, the effects of a CLA/telmisartan combination are unknown. In this 20 wk study, dietary CLA (1.0% w/w) and telmisartan (5 mg/kg body weight per day in drinking water) versus a high-carbohydrate, high-fat control diet were tested for effects on body weight, food and water intakes, lipid profiles, glucose, insulin, blood pressure, certain cytokines, and visceral fat in adult male Sprague-Dawley (SD) rats. Differences in outcomes were detected by one-way ANOVA with post-hoc Tukey tests. Animals treated with CLA/telmisartan combination exhibited significant attenuation in weight gain (550 ± 68 g; $p<0.01$) relative to their counterparts in either control (721 ± 44 g), or losartan- (696 ± 96 g), or CLA-treated (690 ± 90 g) animals; these changes were associated with parallel reduction in visceral fat size, but similarity in food and water intakes. The diet/drug co-administration also resulted in significantly reduced serum total cholesterol (TC) (1.6 ± 0.5 mmol/L vs. 2.4 ± 0.3 mmol/L; $p<0.05$), triglyceride (TG) (1.1 ± 0.2 mmol/L vs. 3.8 ± 0.7 mmol/L; $p<0.001$), and glucose (6.6 ± 0.5 mmol/L vs. 8.6 ± 0.6 mmol/L; $p<0.01$) concentrations in treated rats as compared to the controls. Moreover, CLA/telmisartan significantly decreased serum TC levels as compared to losartan-treated rats ($p<0.05$), and TG levels as compared to either losartan- or CLA-treated rats

($p<0.05$). Lower systolic blood pressure (SBP) was induced by the combination therapy when compared to placebo (121.0 ± 13.0 mm Hg vs. 138.0 ± 8.0 mm Hg; $p<0.05$, respectively). Plasma insulin concentrations were reduced by the combination protocol (1.2 ± 0.4 ng/ml vs. 2.3 ± 0.4 ng/ml; $p<0.01$) relative to the controls and losartan-treated rats ($p<0.05$) whereas no notable differences in leptin levels were observed. Plasma levels of IL1-α and IFN-γ were reduced by 20% and 6%, respectively, in CLA/telmisartan-treated rats when compared to the controls whereas levels of IL1-β, IL-4, IL-6, IL-10, and TNF-α were increased by 28%, 55%, 36%, 75%, and 188%, respectively.

Data of this study suggest that a combination of CLA with telmisartan is 1) safe and well-tolerated in rats over 20 weeks, 2) may attenuate high body weight, reduce hyperlipidemia and hypertension, improve glucose, and normalize insulin metabolism, and thus 3) may prevent or delay onset of MetS in SD rats and improve cardiovascular risk, and 4) may represent an effective, and safe, novel approach in the management of MetS in humans. These findings should motivate future studies to investigate the mechanisms behind the observed effects of the CLA/telmisartan co-therapy.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ACAT	Acyl-Coenzyme A cholesterol acyltransferase
Apo	Apolipoprotein
ARB	Angiotensin-II-receptor blocker
ATP III	Adult Treatment Panel III
BP	Blood pressure
C/EBPδ	CCAAT/enhancer-binding protein δ
c9,11t CLA	cis-9,trans-11 conjugated linoleic acid
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CNS	Central nervous system
CPT	Carnitin palmitoyltransferase
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
EDTA	Ethylenediamine tetra-acetic acid
FA	Fatty acid
FFA	Free fatty acid
FABP	Fatty acid binding protein
GOD	Glucose oxidase
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
HSL	Hormone-sensitive lipase
IL1-α, -β	Interleukin 1-α, -β
IL-4, -6, -10	Interleukin-4, -6, -10
INF-γ	Interferon-γ
ip	Intraperitoneal
IR	Insulin resistance
KO	Knockout
LBM	Lean body mass
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LXR	Liver X receptor
MetS	Metabolic syndrome
NF-κB	Nuclear factor-κB
OGTT	Oral glucose tolerance test
POD	Peroxidase
PPAR	Peroxisome-proliferator-activated receptor
PPARα	Peroxisome proliferator activated receptorα
PPARβ/δ	Peroxisome proliferator activated receptorβ/δ
PPARγ	Peroxisome proliferator activated receptorγ
PUFA	Polyunsaturated fatty acid
RXR	Retinoid X receptor
SBP	Systolic blood pressure

SD rat	Sprague-Dawley rat
SD	Standard deviation
SPPARM	Selective Peroxisome proliferator activated receptor modulator
SREBP	Sterol regulatory element-binding protein
t10,c12 CLA	trans-10,cis-12 conjugated linoleic acid
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
TNF-α	Tumor necrosis factor–α
TRLs	Triglyceride-rich lipoproteins
TZD	Thiazolidinedione
VLDL	Very low density lipoprotein

1.0 INTRODUCTION

Recent epidemiological studies have shown that the prevalence of metabolic syndrome (MetS) is increasing worldwide owing to lifestyle changes that promote obesity (Ginsberg 2003; Aude et al. 2004; Deen 2004). The term metabolic syndrome describes an observed constellation of multiple related clinical disorders that appears to be of major importance in today's pathogenesis of cardiovascular disease (CVD) and diabetes (Moller 2005; Reaven 2005). The cluster of abnormalities that define MetS includes insulin resistance (IR) or glucose intolerance, central obesity, atherogenic dyslipidemia, elevated blood pressure or hypertension, and a pro-inflammatory state (Reaven 1988; Moller 2005; Reaven 2005). Differences in genetic background, physical activity, age, gender, and dietary approaches influence the prevalence of the MetS. Both lifestyle modification (i.e. weight reduction and dietary interventions) and therapeutic agents (i.e. anti-obesity, insulin-sensitizing, lipid-lowering, and anti-hypertensive drugs) have been proposed for the management of MetS (Aude et al. 2004; Daskalopoulou et al. 2004; Hawley et al. 2004; Mazzone 2004; Sowers et al. 2004).

Owing to the untoward effects and cost of therapy of the polypharmacy, it is desirable to develop single drugs that can control multiple risk factors of MetS. The peroxisome proliferator-activated receptor (PPAR) agonists and mixed agonists of various PPAR isoforms (α, β/δ, and γ) have emerged as potential candidates for the prevention and treatment of MetS. Upon binding to the response elements, members of this family of transcription factors either

repress or induce transcription of target genes. Consequently, the resultant changes in gene expression account for effects on lipid, glucose, and energy metabolism (Ramachandran et al. 2006). PPARs can be modulated by natural (i.e. fatty acids [FAs] and FA metabolites) and synthetic (i.e. lipid-lowering and anti-diabetic) compounds.

The discovery of the omega-6 FA conjugated linoleic acid (CLA), primarily in ruminant meat and dairy products, in 1987 led researchers to examine its beneficial effects on cancer (Ha et al. 1990), immune function (Miller et al. 1994), atherosclerosis (Lee et al. 1994), weight gain and food intake (Chin et al. 1994), and body composition (Park et al. 1997). On the basis of these studies, and others (Devery et al. 2001; Pariza et al. 2001; Nicolosi et al. 1997; West et al. 1998; DeLany et al. 1999; Park et al. 1999a; Tsuboyama-Kasaoka et al. 2000; Kritchevsky et al. 2000; Poulos et al. 2001; Blankson et al. 2000; Turek et al. 1998; Hayek et al. 1999; Wong et al. 1997; Yang et al. 2000; Bassaganya-Riera et al. 2001; O'Shea et al. 2004), CLA isomers have been proposed to be able to prevent chemically-induced tumors, protect against catabolic effects of immune stimulation, improve feed efficiency, reduce excess weight, reduce body fat, increase lean body mass, and lower blood lipids in animal models and/or human subjects. On the molecular level, CLA has been shown to modulate the activity of PPARα (Moya-Camarena et al. 1999a; Moya-Camarena et al. 1999b) and PPARγ (Houseknecht et al. 1998; Yu et al. 2002) subtypes in a dose-dependent manner.

Recently, researchers (Benson et al. 2004) have discovered that telmisartan, a selective angiotensin II (AII) type 1 receptor (AT1-R) blocker (ARB) approved for the treatment of essential hypertension, is also a partial agonist of PPARγ (Kurtz and Pravenec 2004; Pershadsingh and Kurtz 2004). Therefore, telmisartan has been proposed to modulate two major metabolic pathways, one through the activation of PPARγ (Jiang et al. 1998; Ricote et al. 1998) pathway, and the other by selectively blocking the AT1-R-dependent pro-inflammatory, pro-atherogenic (Papademetriou 2002; Smith et al. 2004; Suzuki et al. 2003) pathway.

On the basis of our current stage of knowledge, we sought to investigate whether a combination of dietary CLA and the ARB/PPARγ partial agonist telmisartan would synergistically or additively ameliorate a diet-induced MetS in the Sprague-Dawley (SD) rat model.

2.0 REVIEW OF THE LITERATURE

2.1 THE METABOLIC SYNDROME

2.1.1 Definitions, History, and Prevalence

Starting in the 1960s and 1970s, researchers began to document a clustering of elements of cardiovascular risk in certain patients. It was not until 1988 that a unifying cause – IR – was proposed and the term Syndrome X applied. After several name changes over the past two decades, the term metabolic syndrome (MetS) was chosen, by most researchers, to describe the entity.

The syndrome originally included resistance to insulin-stimulated glucose uptake, glucose intolerance, hyperinsulinemia, hypertension, dyslipidemia characterized by high triacylglycerol (TG) concentrations, and low concentrations of high-density lipoprotein (HDL) (Reaven 1988). More recently, the list of abnormalities has been expanded to include central obesity (Purnell et al. 1997); presence of small, dense low-density lipoprotein (LDL) particles (Reaven et al. 1993); increased plasma uric acid concentrations (Reaven 1995); higher circulating concentrations of plasminogen activator-inhibitor (Reaven 1997); decreased circulating concentrations of adiponectin (Havel 2002); and a pro-inflammatory state (Tamakoshi et al. 2003). **Figure 1** summarizes the abnormalities of MetS.

In an effort to introduce the MetS into clinical practice, several organizations have attempted to formulate simple criteria for its diagnosis (**Table 1**). The development of an internationally recognized definition for the

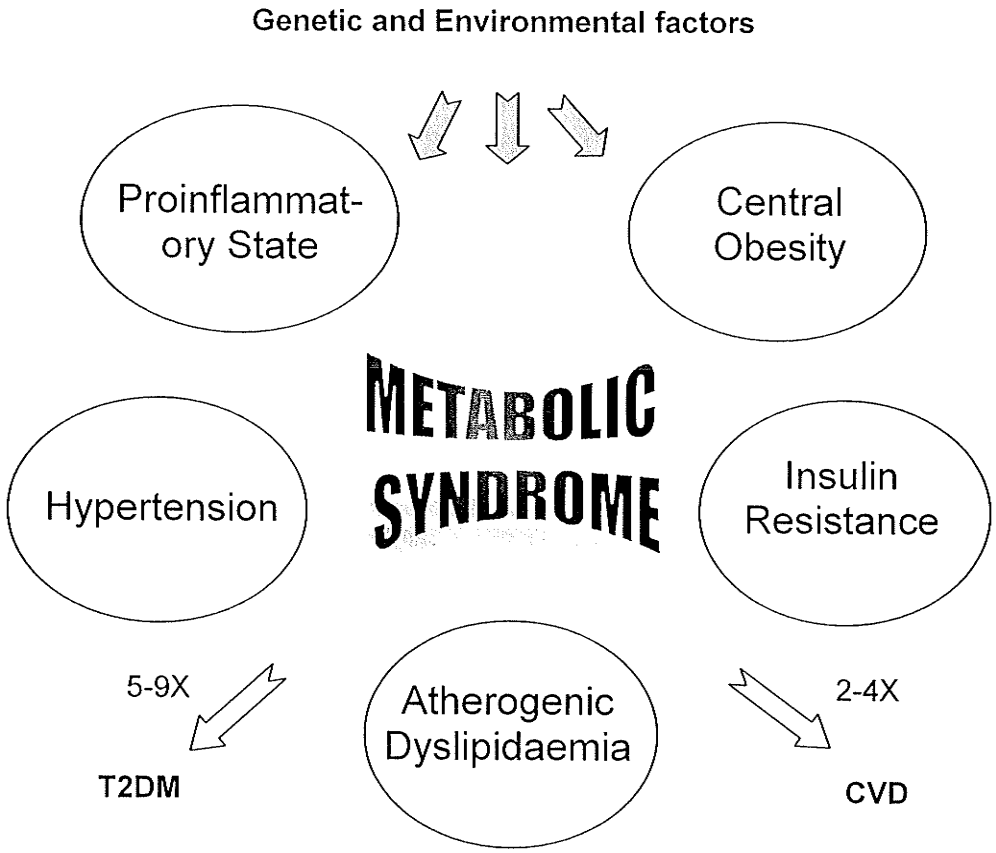


Figure 1. Characteristics of the metabolic syndrome (MetS).

The major risk factors of MetS are central obesity, insulin resistance, atherogenic dyslipidaemia, hypertension, and a pro-inflammatory state; each risk factor has several components. The etiology of MetS is unknown but presumably represents a complex interaction between genetic and environmental factors including diet. Risk for type 2 diabetes mellitus (T2DM) is increased by 5-9 folds, and for cardiovascular disease (CVD) is increased by 2-4 folds, in patients with MetS.

Table 1. Proposed Criteria for Clinical Diagnosis of Metabolic Syndrome.

Clinical Measure	WHO, 1998	EGIR, 1999	ATP III, 2001	AACE, 2003	IDF, 2005
Insulin resistance	T2DM or IFG or IGT or IR **plus 2 of the following**	IR; top 25% fasting insulin **plus any 2 of the following**	None but **any 3 or more of the following**	IGT or IFG **plus any of the following**	None
Obesity/central obesity	BMI > 30 kg/m^2 or waist-to-hip ratio >0.90 (male) or >0.85 (female)	WC ≥94 cm (male) or ≥80 cm (female)	WC ≥102 cm (male) or ≥88 cm (female)	BMI ≥25 kg/m^2	Increased WC (population specific) **plus any 2 of the following**
Dyslipidaemia	TG ≥1.7 mmol/L and/or HDL-C <0.9 mmol/L (male) or <1.0 mmol/L (female)	TG ≥2.0 mmol/L and/or HDL-C <1.0 mmol/L (male and female)	TG ≥1.7 mmol/L and/or HDL-C <1.0 mmol/L (male) or <1.3 (female)	TG ≥1.7 mmol/L and/or HDL-C <1.0 mmol/L (male) or <1.3 (female)	TG ≥1.7 mmol/L and/or HDL-C <1.0 mmol/L (male) or <1.3 (female)
Hypertension	≥140/90 mm Hg	≥140/90 mm Hg and/or medication	≥130/85 mm Hg or medication	≥130/85 mm Hg	≥130/85 mm Hg or medication
Glucose/diabetes	IGT, IFG, or T2DM	Fasting glucose ≥6.1 mmol/L (but not diabetes)	Fasting glucose ≥6.1 mmol/L (includes diabetes)	Fasting glucose ≥6.1 mmol/L (but not diabetes)	Fasting glucose ≥6.1 mmol/L (includes diabetes)
others	Microalbuminuria			Any features of IR	

T2DM, type 2 diabetes mellitus; WC, waist circumference; BMI, body mass index; TG, triglycerides; IGT, impaired glucose tolerance; and IFG, impaired fasting glucose.

syndrome was not initiated until 1998 (Eckel et al. 2005) when a World Health Organization (WHO) consultation proposed a set of criteria to define the MetS (Alberti and Zimmet 1998). Subsequently, the European Group for Study of Insulin Resistance (EGIR) (Balkau and Charles 1999) and the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) (2002) formulated their own definitions in 1999 and 2001, respectively. In 2003, the American Association of Clinical Endocrinologists (AACE) modified the ATP III criteria to refocus on IR as the primary cause of the metabolic risk factors (Einhorn et al. 2003). More recently, the International Diabetes Foundation (IDF) published new criteria that again modified the ATP III definition (2005). Currently, the American Heart Association and the National Heart, Lung, and Blood Institute (AHA/NHLBI) statement maintains the ATP III criteria except for minor modifications.

MetS is known to be prevalent both in developed and developing countries. Approximately 25% of the Canadian adult population is believed to be affected (Anand et al. 2003). Similarly, the age-adjusted prevalence among adults in the United States has been estimated at 24% (Ford et al. 2002).

2.1.2 Components of Metabolic Syndrome

2.1.2.1 Insulin Resistance

The most accepted and unifying hypothesis to describe the pathophysiology of the MetS is the IR. IR has traditionally been defined with a glucocentric view — i.e. when a defect in insulin action results in fasting

hyperinsulinaemia to maintain euglycaemia. Yet, even before fasting hyperinsulinaemia develops, postprandial hyperinsulinaemia exists.

A major contributor to the development of IR is an overabundance of circulating FAs. Plasma free FAs (FFAs) are derived mainly from adipose tissues to the circulation through the action of hormone-sensitive lipase (HSL), or the lipoprotein lipase (LPL) (Eckel et al. 1989). Insulin is important for both antilipolysis and the stimulation of LPL. When IR develops, the increased amount of lipolysis of stored TG molecules in adipose tissue produces more FAs, which further inhibit the antilipolytic effect of insulin, creating additional lipolysis. Elevated levels of FAs may contribute to IR by the added substrate availability and by modifying downstream signaling (Eckel et al. 2005).

Circulating FFAs increase hepatic glucose production and diminish insulin activity (Boden and Shulman 2002), and were found to stimulate target genes of sterol response element binding proteins (SREBPs), transcription factors that stimulate lipid synthesis (Shimomura et al. 1999).

2.1.2.2 Glucose Intolerance

The defects in insulin action in glucose metabolism include deficiencies in the ability of the hormone to suppress glucose production by the liver and kidney, and to mediate glucose uptake and metabolism in insulin sensitive tissues (i.e. muscle and adipose tissue). The relation between impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) and IR is well supported by human, non-human primate, and rodent studies. To compensate for defects in

insulin action, insulin secretion and/or clearance must be modified to sustain norlam glucose levels. If this compensation fails, defects in insulin secretion predominate. Although FFAs can stimulate insulin secretion, prolonged exposure to excessive concentrations of FAs results in a decrease in insulin secretion (Lee et al. 1994). The mechanism for this alteration has been attributed to lipotoxicity through several potential different mechanisms (Yaney and Corke 2003; Boucher et al. 2004; Joseph et al. 2004). Insulin can also feedback on its own secretion. When the insulin receptor is deleted in skeletal muscle, hyperglycaemia does not result (Bruning et. al. 1998), however, the cell specific knockout of the insulin receptor produces progressive glucose intolerance and diabetes (Kulkarni et al. 1999).

2.1.2.3 Central Obesity

The prevalence of obesity has doubled in the past 20 years (Flegal et al. 2002) and became a major public health problem worldwide. The World Health Organization (WHO) estimates that there are globally more than 1 billion overweight adults, 300 million of whom are obese (2005). Also, according to the Canadian Institutes of Health Research (CIHR), 23% of Canadian adults were considered obese in 2004, compared to 14% in 1978 (2004). Further, it is estimated that at any given time, approximately 45% of women and 30% of men are trying to lose weight in the United States (Serdula et al. 1999).

Both genetic and environmental factors have a major impact on the metabolic and cardiovascular consequences of obesity. A major challenge for MetS research remains the identification of adiposity features that best reflect the increased risk of developing the MetS (Reilly and Rader 2003). Although the first description of MetS was in the early 20th century (Eckel et al. 2005), the worldwide obesity epidemic has been the most important driving force in the much more recent recognition of the syndrome. Despite the importance of obesity in the model, it should be mentioned that patients of normal weight can also be insulin resistant (Ruderman et al. 1998).

2.1.2.4 Atherogenic Dyslipidaemia

In general, with increases in FFAs flux to the liver, increased production of apo B-containing triglyceride-rich very low-density lipoproteins (VLDL) occurs (Lewis et al. 1995). The effect of insulin on this process is somewhat complex. In the setting of IR, increased flux of FFAs to the liver increases hepatic TG synthesis; however, under physiological conditions, insulin inhibits rather than increases the secretion of VLDL into the systemic circulation (Lewis and Steiner 1996). This response in part is an effect of insulin on the degradation of apo B (Taghibiglou et al. 2002). Yet, insulin is also lipogenic, increasing the transcription of genes activity of enzymes that relate to TG biosynthesis (Foufelle and Ferre 2002). Whether or not this pathway remains operational in the setting of systemic IR has yet to be completely addressed. Additionally, IR could reduce the concentrations of LPL in peripheral tissues

(in adipose tissue more than muscle) (Eckel et al. 1995). This alteration in LPL, however, seems to contribute less to the hypertriglyceridaemia than does the overproduction of VLDL. Nevertheless, hypertriglyceridaemia reflects the insulin resistant condition and is one of the important criteria for diagnosis of the MetS.

2.1.2.5 Hypertension

High blood pressure is prevalent both in obesity and diabetes (Ferrannini et al. 1987). The relation between IR and hypertension is also well established (Ferrannini et al. 1987) and relates to several different mechanisms. First, insulin is a vasodilator when given intravenously to people of normal weight (Steinberg et al. 1994), with secondary effects on sodium reabsorption in the kidney (DeFronzo et al. 1975). In the setting of IR, the vasodilatory effect of insulin can be lost (Tooke and Hannemann 2000), but the renal effect on sodium reabsorption preserved (Kuroda et al. 1999). FAs may also contribute to hypertension by causing vasoconstriction (Tripathy et al. 2003). Regardless, when assessed by concentrations of fasting insulin, IR contributes only modestly to the increased prevalence of hypertension in the MetS (Hanley et al. 2002).

2.1.2.6 Pro-inflammatory State

The association of the MetS with inflammation is well documented (Han et al. 2002; Esposito et al. 2003). The increases in pro-inflammatory cytokines

including interleukin-6 (IL-6), resistin, tumour necrosis factor-α (TNF-α) and C-reactive protein (Fernandez-Real and Ricart 2003) reflect overproduction by the expanded adipose tissue mass (Trayhurn and Wood 2004). Evidence suggests that monocyte-derived macrophages reside in adipose tissue, might be, at least in part, the source of both local and systemic pro-inflammatory cytokines (Weisberg et al. 2003; Xu et al. 2003). There is increasing evidence that IR in the liver, muscle, and adipose tissue is not only associated with the abundance of pro-inflammatory cytokines (and relative deficiency of the anti-inflammatory cytokine adiponectin), but is also a direct result of this burden (Neuschwander-Tetri and Caldwell 2003).

2.1.3 Management of Metabolic Syndrome

Although MetS appears to be more common in people who are genetically susceptible, acquired underlying risk factors — overweight or obesity, physical inactivity, and atherogenic diets — commonly elicit clinical manifestations. Clinical trials should first focus on management of these underlying risk factors independent of an individual's risk status (Eckel et al. 2005).

2.1.3.1 Lifestyle and Dietary Interventions

Weight reduction deserves first priority in individuals with abdominal obesity and MetS (Grundy et al. 2006). Both weight reduction and

maintenance of a lower weight are best achieved by a combination of reduced caloric intake and increased physical activity and the use of principles of behavior change. In clinical trials, the first aim of weight loss is to achieve a decline of about 7% to 10% from baseline total body weight during a period of 6 to 12 months. This will require decreasing caloric intake by 500 to 1000 calories per day. Greater physical activity helps to enhance caloric deficit. Achieving the recommended amount of weight loss might well reduce the severity of most or all of the metabolic risk factors (Grundy et al. 2006).

Increasing physical activity also assists in weight reduction and reduces overall CVD risk (Franklin et al. 2004). Current recommendations call for the accumulation of ≥30 minutes of moderate-intensity exercise on most or, preferably, all days of the week (Grundy et al. 2004; Thompson et al. 2003). Going beyond current recommendations will be particularly beneficial for people with the MetS. For example, 60 minutes or more of continuous or irregular aerobic activity will further promote weight loss or weight-loss maintenance (Grundy et al. 2004). Short bouts of activity are also preferable (i.e. walking breaks at work, gardening, or household work), simple exercise (e.g. by treadmills), jogging, swimming, biking, golfing, team sports, and engaging in resistance training (Pollock et al. 2000). Avoiding common sedentary activities in leisure time (i.e. television watching) is also advised.

Beyond weight control and reduction of total calories, the diet should be low in saturated fats, trans fats, cholesterol, sodium, and simple sugars (Krauss et al. 2000). In addition, there should be ample intakes of fruits,

vegetables, and whole grains; fish intake should be encouraged with recognition of concerns about the mercury content of some fish (Chobanian et al. 2003; Kris-Etherton et al. 2002). Very high carbohydrate intakes can exacerbate the dyslipidemia of the MetS. ATP III (2003) recommended that for individuals entering cholesterol management, the diet should contain 25% to 35% of calories as total fat. If the fat content exceeds 35%, it is difficult to sustain the low intakes of saturated fat required to maintain a low LDL-cholesterol. On the other hand, if the fat content falls below 25%, TG can rise and HDL-cholesterol levels can decline (Garg et al. 1994); thus, very-low-fat diets may exacerbate atherogenic dyslipidemia. To avoid worsening of dyslipidemia in patients with MetS, some investigators favor fat intakes in the range of 30% to 35%; others are concerned about possible weight gain resulting from long-term ingestion of higher fat intakes and thus prefer intakes in the range of 25% to 30% (Grundy et al. 2005).

For many years, a low-fat diet was advocated because the high caloric density of fat could increase the likelihood of obesity. More recently, interest has grown in the possibility that high-protein, low-carbohydrate diets will enhance weight reduction (Foster et al. 2003). The rationale seems to be that fat and protein offer satiety that is absent with carbohydrates. That this effect of fat and protein on satiety makes the diet more effective for producing weight loss is an arguable hypothesis. Moreover, research documenting long-term maintenance of a lower body weight that high-fat/high-protein/low-carbohydrate diets can achieve is lacking. In fact, after 1 year of consumption

of low-carbohydrate diets, severely obese patients show no more weight reduction than those eating a conventional weight-loss diet (Stern et al. 2004). High-fat diets not only tend to be higher in saturated fat but they often are deficient in fruits, vegetables, and whole grains — all of which are important components in currently recommended diets. Finally, preoccupation with macronutrient composition to promote weight loss fails to identify the key factors affecting body weight. On the whole, it seems that effective weight loss requires a combination of caloric restriction, physical activity, and motivation; effective lifelong maintenance of weight loss essentially requires a balance between caloric intake and physical activity.

2.1.3.2 Drug Therapy

Beyond lifestyle therapies directed toward underlying risk factors, attention must be given to the metabolic risk factors of MetS. In the case of CVD or diabetes, drug therapies for management of the risk factors may be required (Chobanian et al. 2003; Genuth et al. 2003).

2.1.3.2.1 Anti-Obesity

At present, multiple mechanisms are being evaluated as potential targets for the development of anti-obesity drugs. Although such agents are generally viewed as weight-loss drugs, they could be directed against sites of excess fat storage. Adipose tissue has been shown to be a source of many

products that, in one way or another, can worsen the MetS (Berg and Scherer 2005; Matsuzawa 2005; Trayhurn and Wood 2004).

Most drugs currently being evaluated for weight reduction modify pathways in the central nervous system (CNS). Sibutramine (Meridia®) is one such agent that has been approved for weight reduction. Over about 1 year of testing, sibutramine has been shown to produce weight reduction in a range of 5–10% of total body weight (Arterburn et al. 2004). The drug, however, has shown tendency to raise BP, which is of concern for patients with MetS.

Other candidates for weight reduction are drugs that primarily act outside the CNS. Orlistat (Xenical®), for example, acts entirely in the intestine to inhibit pancreatic and gastric lipases (Curran and Scott 2004), and therefore blocks the absorption of fat. Other agents under consideration enhance energy expenditure by, for instance, causing energy uncoupling in muscle. Included in this group are β3-adrenoceptor agonists, thyroid hormone receptor β-subtype agonists, and peroxisome proliferator- activated receptorγ (PPARγ) agonists.

2.1.3.2.2 Anti-Diabetic

Drugs that are effective for reducing hyperglycaemia can be registered for the treatment of patients with diabetes. Unfortunately, available anti-diabetic drugs have limited efficacy against hyperglycaemia, and consequently multiple drugs of this type are required for glucose control.

One oral hypoglycaemic agent that could have a modest effect on other risk factors is metformin, the primary action of which seems to be lowering hepatic glucose output, which decreases IR and plasma glucose levels

(Gerich 1995). This action has been attributed to the activation of AMP kinase (Zhou et al. 2001). In one large study in patients with T2DM, metformin therapy lowered risk for major coronary events (UKPDS 1998). The drug might, in some way, act to alleviate MetS as a whole (Adler et al. 1998). Metformin has been available for many years; it is relatively inexpensive and widely used for the treatment of diabetes worldwide. Moreover, at least one combination of metformin with a sulphonylurea has been introduced to improve anti-hyperglycaemic therapy. If future clinical trials could prove that metformin reduces risk for CVD, this would make it a preferred agent for the treatment of T2DM, and perhaps as a monotherapy even for the treatment of the MetS without diabetes.

Another potentially efficacious class of drugs for the MetS as a whole is the thiazolidinediones (TZDs). These drugs act by agonizing the nuclear receptor PPARγ, which is predominantly expressed in adipose tissue, but also occurs in other tissues (Jiang et al. 2002; Way et al. 2001). TZDs reduce the secretion of unesterified FAs and adipokines such as TNFα, and resistin. They also enhance adipose-tissue release of adiponectin (Staels 2005). The net result of these changes is to reduce IR in muscle and the liver and to mitigate the proinflammatory state. Based on these findings, it has been suggested that TZDs are hitting at the heart of the MetS by improving IR in adipose tissue. Still, in T2DM, TZDs have only a modest effect on plasma lipoproteins and BP. So, although they improve the MetS, they do not cure it once T2DM develops. Pioglitazone (Actos®) and rosiglitazone (Avandia®) are two PPARγ agonists

currently available. Other, more potent, TZDs have been evaluated but were accompanied by increased side effects that have hindered their registration. Currently, a combination formulation of pioglitazone plus metformin has received regulatory approval, suggesting a trend towards therapeutic generalization through drug combinations. Weight gain, fluid retention, and an increase in total body fat are among major side effects of the TZDs (Tang 2006).

2.1.3.2.3 Lipid-Lowering and Anti-Atherogenic

Lifestyle therapies are believed to improve all of the lipoprotein abnormalities in MetS, but when lifestyle modification does not fully correct them, consideration has to be given to drug treatments. Several drugs are currently available to treat high apo B levels. Although these drugs, in general, do not affect other metabolic risk factors, they nonetheless have considerable potential to reduce risk for CVD. Key agents to treat elevated apo B and/or atherogenic dyslipidaemia are 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (i.e. statins), cholesterol-absorption blockers, bile-acid sequestrants, nicotinic acid, and PPARα agonists (fibrates). Several other lipid-lowering drugs are also under development, an example of which is the cholesteryl ester transfer protein (CETP) inhibitors (Grundy et al. 2006).

2.1.3.2.4 Anti-Hypertensive

Currently, many drugs are registered for the treatment of hypertension. The majority of individuals with the MetS have some elevation of BP, and

when T2DM ensues, hypertension is almost always present. Although lowering BP reduces cardiovascular risk, efficacy of single-drug therapy is limited, and even multi-drug treatment often does not normalize BP in hypertensive patients. The necessity for combining several anti-hypertensive agents in most patients with hypertension, including those with MetS and T2DM, exacerbates the polydrug predicament. Moreover, which anti-hypertensive drugs are most efficacious for patients with the MetS is still a matter of some debate.

Diuretics and beta-blockers, two of the older drugs used for the treatment of hypertension, are still widely used in clinical practice. Both have been reported to worsen IR and dyslipidemia (Pollare et al. 1989; Lithell 1991) making these agents less attractive for managing the MetS.

Another class of anti-hypertensives, the calcium channel blockers, seems to be neutral with respect to both lipids and IR. Conversely, $\alpha 1$ blockers and imidazoline receptor agonists (i.e. moxonidine) have been reported to increase insulin sensitivity (Lithell 1997). Moxonidine and a related drug, rilmenidine, inhibit sympathetic outflow and cause vasodilation which, in turn, enhances peripheral insulin action and, thereby, reduces IR. Drugs of this type could hold promise for the treatment of the MetS.

A final group of agents that lower BP interfere with the renin–angiotensin–aldosterone system (RAAS). Angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) are two types of agents that affect the RAAS. Beyond blood-pressure lowering, these drugs

might have beneficial actions on metabolism, inflammation, and vascular biology. If so, they could be particularly beneficial for patients with MetS. Although not consistent for all studies, some investigators report that ACEIs and ARBs reduce IR (Julius et al. 2001). Yet, several clinical trials suggest that ACEIs and ARBs reduce the risk for developing T2DM in patients with pre-diabetes (Jandeleit-Dahm et al. 2005). In addition, various mechanisms have been postulated by which ACEIs or ARBs will reduce progression of atherosclerosis, including improvement of endothelial function, favorable changes in fibrinolytic balance, enhanced nitric oxide (NO) release, and reduced vascular inflammation (Nickenig 2004; Lonn 2002).

So far, there has been evidence that at least one anti-hypertensive agent, the ARB telmisartan (Micardis®), may have unique properties for reducing risk for cardiovascular events beyond BP lowering (Benson et al. 2004). The PPARγ partial agonist telmisartan is discussed in more detail subsequently in this dissertation.

Obviously, as the number of drugs required to better control risk factors and complications of MetS increases, so do the possibilities of untoward effects — drug side effects, drug–drug interactions, failure of adherence, and medication errors — and so does the cost of therapy (Rollason et al. 2003). Therefore, pharmaceutical industry has shown great interest in designing drugs that can target the MetS as a whole, which could mitigate polypharmacy.

2.2 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

During the last decade, it has been shown that pharmacological activations of PPARs are effective therapeutic approaches to correct some aspects of MetS. Several observations revealed that these beneficial actions of PPARs activators are related, at least in part, to their effects on plasma lipid levels. PPARs are members of the nuclear receptor superfamily that includes steroid and thyroid hormones (Francis et al. 2003) and consists of a group of three isoforms: PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3). PPARs bind to the peroxisome proliferator response element (PPRE) and repress or induce the transcription of the target genes. The resultant changes in gene expression account for effects on lipid metabolism, energy balance, thermogenesis, glucose metabolism, as well as atherosclerotic and the carcinogenic processes (Ramachandran et al. 2006; Savkur and Miller 2006). While the best characterized action of PPARα is to mediate the uptake and oxidation of FAs in various tissues including the liver and the heart (Puddu et al. 2003; Bishop-Bailey 2000), PPARβ/δ is best known for its role in skin homeostasis, and has recently shown a role in HDL metabolism (Francis et al. 2003; Bishop-Bailey 2000). PPARγ has an ability to promote preadipocyte differentiation, mediate lipogenesis in adipocytes, and enhance insulin sensitivity (Francis et al. 2003; Spiegelman 1998).

A variety of novel PPAR approaches are currently being investigated for the treatment of T2DM and MetS, including the highly potent selective PPAR

agonists and PPAR combination agonists (Miller and Etgen 2003). PPARα activators, such as the normolipidaemic fibrates, decrease TG concentrations by increasing the expression of LPL and decreasing apo C-III concentration (Francis et al. 2003). Furthermore, they increase HDL-cholesterol by increasing the expression of apo A-I and apo A-II (Francis et al. 2003). PPARα activation by fibrates improves insulin sensitivity and decreases thrombosis and vascular inflammation (Francis et al. 2003a). Clinical studies with bezafibrate and gemfibrozil have shown significant protective effects against CVD (Fruchart et al. 2001; Vosper et al. 2002). Activation of PPARγ improves insulin sensitivity and decreases inflammation, plasma levels of FFAs and BP. This leads to the inhibition of atherogenesis, improvement of endothelial function, and reduction of cardiovascular events (Francis et al. 2003a). The TZD group of PPARγ ligands, such as rosiglitazone and pioglitazone, have beneficial effects on serum lipids in diabetic patients and have also been shown to inhibit the progression of atherosclerosis in animal models (Gurnell et al. 2003). Recent studies have found that PPARβ/δ is also a regulator of serum lipids (Michalik et al. 2003). However, there are currently no drugs in clinical use that selectively activate this receptor. Therefore, modulators that promote both PPARα and/or PPARγ agonism could theoretically offer significant benefits in improving the dyslipidaemia state, reducing hyperglycaemia, and, thus, hinder the CVD risk factors associated with the MetS. In addition, such a therapy could improve the underlying IR and help

break the cycle of altered glucose and lipid metabolism that promotes T2DM (Bishop-Bailey and Wray 2003).

2.2.1 PPARs and Adipogenesis

PPARγ is the foremost regulator of adipogenesis (Rosen et al. 2000; Rosen and Spiegelman 2001). It is highly expressed in adipose tissue, with induced expression early in the preadipocyte differentiation (Saladin et al. 1999). Studies modulating PPARγ expression or action in rodent cell lines have confirmed that the receptor is essential, and the presence of PPARγ agonists is sufficient for adipogenesis (Rosen and Spiegelman 2001; Rosen et al. 2002).

Activation of PPARα by FAs promotes hepatic FA oxidation to generate ketone bodies, providing an energy source for peripheral tissues. This regulation is important, as PPARα-null mice are unable to meet energy demands during fasting and suffer from hypoglycemia, hyperlipidemia, hypoketonemia and fatty liver (Kersten et al. 1999). In a variety of mouse models, PPARα-agonists lower reduce adiposity, improve hepatic and muscle steatosis, and consequently improving insulin sensitivity (Guerre-Millo et al. 2000; Chou et al. 2002; Kim et al. 2003).

2.2.2 PPARs and Insulin Sensitivity

Studies of TZDs have provided strong evidence for the role of PPARγ in insulin sensitivity (Lehmann et al. 1995; Hulin et al. 1992). TZDs have been shown to control blood glucose via an insulin-sensitizing mechanism in animal models of T2DM and in human subjects (Day 1999). Lehmann et al. (1995) have reported that TZDs are selective high-affinity ligands for PPARγ. Moreover, selective non-TZD PPARγ ligands have been recently shown to exert potent anti-diabetic effects (Willson et al. 2000; Fiedorek et al. 2000) in human subjects and animal models. The severe hypoglycemia observed specifically in PPARα-deficient mice upon fasting, characterized by a 50% drop in blood glucose concentration after 24 hours of fasting, suggested a role for PPARα in glucose homeostasis (Kersten et al. 1999). Several mechanisms may account for this fasting hypoglycemia (Bandsma et al. 2004; Xu et al. 2002). Also, PPARα-selective agonists have been shown to to improve insulin sensitivity and to reduce adiposity (Guerre-Millo et al. 2000; Koh et al. 2003).

2.2.3 PPARs and Dyslipidaemia

Failure of insulin to suppress lipolysis leads to inappropriate delivery of FFAs from peripheral tissues to the liver, in which they are stored as triglyceride and/or packaged into VLDLs. This leads to elevated blood triglyceride levels with subsequent lowering of circulating HDL-cholesterol levels. It is now believed that subjects with loss-of-function mutations in the

PPARγ will have high triglycerides and LDL-cholesterol, and low HDL-cholesterol levels (Barroso et al. 1999; Agarwal and Garg 2002; Hegele et al. 2002; Savage et al. 2003). PPARγ agonists, which have been shown to improve insulin sensitivity, would be expected to lower circulating TG and raise HDL-cholesterol concentrations. On the other hand, activation of PPARα has been long known to induce several pathways affecting lipid metabolism in the liver: increased FA oxidation; reduced production of apolipoprotein CIII (an inhibitor of LPL); and increased production of apolipoprotein AI (Gervois et al. 2001). The primary clinical use of the three currently available PPARα agonists fibrates — gemfibrozil, fenofibrate, and bezafibrate — is to treat atherogenic dyslipidaemia.

2.2.4 PPARs and Hypertension

Several studies have demonstrated that early-onset hypertension is a prominent clinical finding in individuals with PPARγ mutations (Barroso et al. 1999; Agarwal et al. 2002). In a few clinical trials, TZDs were shown to effectively reduce BP in patients with T2DM and hypertension, nonhypertensive type 2 diabetics, obese subjects without diabetes, and nondiabetic hypertensives (Parulkar et al. 2001).

2.2.5 PPARs and Inflammation

PPARs are FA receptors that are widely expressed in the immune system and engaged in regulating expression of various genes involved in proliferation of lymphocytes and monocytes or macrophages, apoptosis, and inflammation. In particular, synthetic PPARγ agonists are known to inhibit the production of proinflammatory cytokines, including TNFα in monocytes (Jiang et al. 1998), in addition to affecting the differentiation of monocytes and macrophages (Tontonoz et al. 1998). PPARα agonists, on the other hand, have been reported to repress NF-κB and activator protein 1 signalling (Staels and Fruchart 2005), and in various cellular systems fibrates modify activities or expression of endothelin 1 in arterial endothelium (Delerive et al. 1999), interleukin-6, cyclooxygenase-2, nitric oxide (NO) synthase (Staels and Fruchart 2005), tissue factor (Marx et al. 2001; Neve et al. 2001) and fibrinogen. Other activities that could be anti-inflammatory or anti-atherogenic are being explored (Staels and Fruchart 2005).

2.3 CONJUGATED LINOLEIC ACID

CLA has been a subject of considerable research over the past two decades. CLA may represent an exciting new horizon in the science of FAs and human health. The discovery of numerous positive physiological effects of CLA has generated an increased interest worldwide.

2.3.1 Definition and Historical Review

CLA is a naturally occurring essential FA that is primarily found in beef and dairy fats. It can also be produced in the laboratory and through manufacturing processes that convert natural linoleic acid (LA), an omega-6 FA, into CLA. Despite being an omega-6, CLA shares several biological activities with omega-3 FA including the cardiovascular benefits.

CLA research dates back to 1950s and 1960s (Reiser 1950; Scott et al. 1959). However, it was not until 1987 that it became the core of interest when Michael Pariza and colleagues at Food Research Institute in the University of Wisconsin made their observation that CLA, isolated from grilled beef, inhibits the chemically-induced skin neoplasia in mice (Ha et al. 1987). This discovery led the group to a flurry of research examining CLA's beneficial effects on cancer (Ha et al. 1990), immune function (Miller et al. 1994), atherosclerosis (Lee et al. 1994), weight gain and food intake (Chin et al. 1994), and body composition (Park et al. 1997). On the basis of these studies, it was concluded that feeding (0.5% w/w) a commercially available crude mixture of CLA isomers may prevent chemically-induced tumors, protect against catabolic effects of immune stimulation, improve feed efficiency, reduce excess weight gain, reduce body fat, increase lean body mass, and lower blood lipid profiles. Other studies support CLA's anti-carcinogenic (Devery et al. 2001; Pariza et al. 2001), anti-atherogenic (Lee et al. 1994; Nicolosi et al. 1997), anti-diabetic (Houseknecht et al. 1998), as well as anti-obesity (Park et al. 1997;

Houseknecht et al. 1998; West et al. 1998; DeLany et al. 1999; Park et al. 1999a; Park et al. 1999b; Tsuboyama-Kasaoka et al. 2000) properties.

2.3.2 Chemistry and Dietary Sources

CLA refers to a mixture of positional and geometric isomers of conjugated dienoic derivatives of LA (Adlof 1999) endogenously generated via the carbon-centered free radical oxidation of LA (Albright et al. 1996). LA is an 18-carbon unsaturated FA with two double bonds in positions 9 and 12. Both of these bonds lie in the "cis" configuration, giving LA its own unique chemical name as cis-9,cis-12 (c9,c12) octadecadienoic acid. The major isomers of CLA differ modestly from LA in that the two double bonds in CLA exist predominantly in one of three positions along the carbon chain: 9 and 11, 10 and 12, or 11 and 13. These small chemical changes not only give CLA its unique chemical name, but due to the varied positions of the double bonds, CLA contains both "cis" and "trans" configurations.

As summarized in **Table 2**, the principal dietary sources of CLA are dairy products such as milk and cheese, and ruminant meats such as beef and lamb (Chin et al. 1992; Lin et al. 1995). The total CLA content in foods can vary substantially, with the cis-9, trans-11 (c9,t11) isomer usually representing the predominant form (Chin et al. 1992). Typically, meat from ruminants contains more CLA than meat from non-ruminants. On the other hand, cheese and dairy products are good sources, while seafood and vegetable oils are not particularly high in CLA (Ip 1994). The c9,t11 isomer accounts for less than

*Table 2. Dietary sources of CLA *.*

Dairy products	*mg/g fat*	Meats/fish	*mg/g fat*
Condensed milk	7.0	Lamb	5.8
Colby	6.1	Fresh ground beef	4.3
Butter fat	6.1	Veal	2.7
Ricotta	5.6	Fresh ground turkey	2.6
Homogenized milk	5.5	Chicken	0.9
Cultured buttermilk	5.4	Pork	0.6
U.S. processed cheese	5.0	Egg yolk	0.6
Mozarella	4.9	Salmon	0.3
Plain yogurt	4.8		
Custard style yogurt	4.8		
Butter	4.7	**Vegetable oils**	
Sour cream	4.6	Safflower oil	0.7
Cottage	4.5	Sunflower oil	0.4
Low fat yogurt	4.4	Peanut	0.2
2% milk	4.1	Olive	0.0
Mediam cheddar	4.1		
Ice cream	3.6		
Parmesan	3.0		
Frozen yogurt	2.8		

** Based on data of Chin et al. (1992), Lin et al. (1995), and the University of Wisconsin Food Research Institute.*

50% of the total CLA in vegetable oils, in contrast to the 80-90% range found in meat and dairy products. In products of cheese and milk, the amount of CLA ranges from 3.6 to 8.0 mg/g lipid and 3.4 to 6.4 mg/g lipid, respectively (Lin et al. 1995). Depending on the animal species, tissue, and diet, CLA content in ruminant meat products varies from 2.7 to 5.6 mg/g lipid (Chin et al. 1992). Butter contains approximately 720 mg/100 g (Britton et al. 1992). Recent studies suggest that biological effects of CLA are isomer-specific, with the two most predominant isomers c9,t11 and t10,c12 being probably the most active, and thus important, CLA isomers (Ritzenthaler et al. 2001).

2.3.3 Physiological Effects

2.3.3.1 Body Composition

Evidence from animal and clinical research supports the available evidence that CLA can favourably affect body composition, resulting in relative or absolute increase in lean body mass (LBM), while decreasing the total body fat. In various animal models and humans, dietary CLA has been shown to decrease the adiposity. For example, rodents fed 1-1.5% CLA as a mixture of c9,t11 and t10,c12 isomers had less body fat and greater lean body mass than control animals (Park et al. 1997; Houseknecht et al. 1998; West et al. 1998, DeLany et al. 1999). The mechanisms proposed were increased lipolysis, increased FA oxidation or reduced FA uptake in adipocytes. When hamsters were fed a hypercholesterolemic diet containing 1% CLA, 0.2% c9,t11 CLA or 1% LA, CLA isomer mix-fed animals had the lowest weight gain (Gavino et al.

2000). In a study in Zucker diabetic fatty (ZDF) rats, dietary intake of 1.5% of CLA (47% c9,t11 and 47.9% t10,c12) decreased weight gain and fat mass, whereas dietary intake of CLA containing 91% c9,t11 had no effect on these parameters, proving that t10,c12 is the isomer responsible for loss of fat mass (Ryder et al. 2001). Feeding Sprague-Dawley (SD) rats 0.25-0.5% of CLA mixture isomers for 5 weeks has reduced fat pad weights without affecting growth rate or food intake (Azain et al. 2000). Even though, Yamasaki *et al.* (1999) found that SD rats fed 1.0-2.0% mixed CLA isomers had no difference in body weight, food intake, or feed efficiency relative to the controls, CLA-treated animals did have lower levels of TG in white adipose tissue, liver, and serum. These data suggest that dietary CLA reduces adiposity and may, thereby, inhibit the development of obesity-related diseases.

Some of the mechanisms suggested to be involved in fat reduction with CLA intake are increased energy expenditure (West et al. 1998; West et al. 2000; Terpstra et al. 2002; Ohnuki et al. 2001), increased fat oxidation (West et al. 1998; Ohnuki et al. 2001), decreased adipocyte size (Tsuboyama-Kasaoka et al. 2000; Azain et al. 2000; Poulos et al. 2001), decreased energy intake (West et al. 1998) and inhibition of enzymes involved in fatty acid metabolism and lipogenesis (Park et al. 2000; Park and Pariza 2001; Bretillon et al. 1999; Takahashi et al. 2002).

2.3.3.2 Insulin Sensitivity/Glucose Tolerance

CLA may play a role in glucose metabolism through its insulin sensitizing effects. In this regard, CLA activates PPARα in the liver, and shares functional similarities to TZDs ligands of PPARγ, which are potent insulin sensitizers. Early evidence for effects of CLA on insulin sensitivity was provided by Houseknecht et al. (1998) who reported that CLA was able to normalize impaired glucose tolerance, and improve hyperinsulinemia in pre-diabetic ZDF rats. Additionally, dietary CLA appeared to increase activation of PPARγ in adipose tissues. Houseknecht et al. (1998) proposed that insulin-sensitizing effects of CLA are due, at least in part, to the activation of PPARγ. Nagao et al. (2003b) showed that CLA attenuated plasma glucose and insulin and prevented hyperinsulinemia by enhancing plasma adiponectin levels and gene expression in white adipose tissue from ZDF rats. Studies suggest that the effects of CLA may be dependent on fat content of the diet. For example, in a study with SD rats, 1.0% of CLA as a mixture of isomers, c9,t11 alone or t10,c12 alone enhanced glucose tolerance and decreased IR compared to control high-fat diet-fed suggesting that both CLA isomers have beneficial effects on IR (Choi et al. 2004).

Two studies have shown that both t10,c12 (Riserus et al. 2002) and c9,t11 (Riserus et al. 2004) isomers may decrease insulin sensitivity in humans at risk for CVD. In contrast, CLA was found to improve insulin sensitivity in young sedentary humans after 8 weeks, which correlated with decreased fasting insulin levels (Eyjolfson et al. 2004).

2.3.3.3 Cardiovascular Health

The influence of CLA on processes related to atherosclerosis and other cardiovascular disorders has been studied *in vivo* and *in vitro*. These studies demonstrated that CLA may improve blood lipid profiles and prevent atherosclerosis (Sher et al. 2003; Kritchevsky et al. 2000; Poulos et al. 2001; Blankson et al. 2000; Belury et al. 1997; Doyle 1998). Anti-oxidant activities of CLA may also play a role (Carew et al. 1987). Additionally, the ability of CLA to reduce serum TG and cholesterol may contribute to a decrease in atherosclerotic plaque formation in rabbits and hamsters fed hypercholesterolemic diets. In atherosclerosis-prone ApoE knockout mice fed 1.0% cholesterol, intake of an 80:20 (c9,t11:t10,c12) CLA blend (1.0%) not only prevented progression, but completely abolished atherosclerosis as compared to controls (Toomey et al. 2006). Data regarding effects of CLA on lipid profiles in SD rats are limited. One recent study by Kloss et al. (2005) reported that CLA supplementation has no significant effects on serum TG concentrations and HDL-cholesterol within 4 weeks. However, TC concentrations were lower in CLA-supplemented rats relative to those in the control group. The underlying mechanisms involved in the anti-atherosclerotic and lipid-lowering effects of CLA, or its individual isomers, have not been adequately addressed. Nonetheless, some of the proposed mechanisms include their role on PPARs and SREBPs. Although both c9,t11 and t10,c12 isomers are ligands for PPARα, results suggest that c9,t11 isomer is the more potent activator of the two (Moya-Camarena et al. 1999a). However, the

recent finding that CLA-fed PPARα null mice have lower plasma TG levels suggests that lipid-lowering effects of CLA may be independent of PPARα (Peters et al. 2001). SREBP-1 isoforms regulate FA and TG synthesis (Pai et al. 1998). Some studies suggest that the c9,t11, but not the t10,c12, isomer positively influences lipid metabolism by reduced synthesis and cleavage of hepatic SREBP-1, which in turn is regulated by the nuclear hormone receptor liver X receptor (LXR) expression (Roche et al. 2002).

Compared to studies in animal models, there have been very few human studies that have evaluated the effects of CLA on risk factors for cardiovascular health. Of these, Benito et al. (2001) reported that there was no change in plasma lipid or lipoprotein levels after intake of 3.9 g CLA/day containing 11.4% c9,t11 isomer and 14.7% t10,c12 isomer. Mougios et al. (2001), however, showed that CLA supplementation (0.7 g/day for 4 weeks) decreased serum TC and TG, but also decreased HDL-cholesterol.

2.3.3.4 Blood Pressure

CLA (50:50) or the t10,c12 isomer, but not c9,t11, has been consistently shown to decrease BP and hypertension in various rat models prone to develop obesity, diabetes and obesity together, or hypertension (Nagao et al. 2003a; Nagao et al. 2003b; Inoue et al. 2004). Of these, Inoue et al. (2004) provided evidence that CLA suppresses development of non-obese essential hypertension in spontaneously hypertensive rats (SHRs) in 4 weeks. Also, increased levels of plasma adiponectin were observed in same study. As

studies indicate that the PPARγ ligands TZDs increase mRNA expression of adiponectin (Maeda et al. 2001), and that CLA activates PPARγ in white adipose tissue in rats (Houseknecht et al. 1998), Inoue et al. (2004) considered CLA's ability to enhance adiponectin mRNA levels to be probably attributable to activation of its promoter through the PPARγ pathway.

2.3.3.5 Immune System

Pro-inflammatory cytokines (i.e. TNF-α, IL-6, IL-1), anti-inflammatory cytokines (like IL-10), eicosanoids (i.e. prostaglandins and leukotrienes) and nitric oxide (NO) are key inflammatory mediators that can be regulated by dietary intake of polyunsaturated fatty acids (PUFAs), including CLA. Several studies suggest that CLA might enhance immune functions in animals (Turek et al. 1998; Hayek et al. 1999; Wong et al. 1997; Yang et al. 2000; Bassaganya-Riera et al. 2001), and humans (O'Shea et al. 2004).

Early during an immune response, cells of immune system (i.e monocytes and macrophages) secrete cytokines to give directions and contributions to inflammatory reactions. Of such, the pro-inflammatory cytokine TNF-α is a key mediator in many chronic immunopathologies, including atherosclerosis, cancer, and obesity. Even though not all studies agree, decreased production of TNF-α by CLA, in humans and animal models, suggests that the immune status is channeled into an anti-inflammatory profile (O'Shea et al. 2004).

One study in SD rats showed that 1.5% CLA in the diet can decrease serum TNF-α irrespective of fat content of the diet (Yamasaki et al. 2003). In contrast, another study by Sugano et al. (2001) in same rat model fed with different diets and 1% CLA for 3–4 weeks found no effect on serum levels of TNF-α and leptin.

2.4 ANGIOTENSIN II RECEPTOR BLOCKERS

In the classical renin-angiotensin system (RAS), circulating renal-derived renin cleaves angiotensinogen, a protein principally synthesized in the liver, to form the inactive angiotenin I (AI). Conversion of AI to anigiotensin II (AII) is promoted by the angiotensin-converting enzyme (ACE) mainly in the lungs (Campbell 2003). There is widespread agreement that RAS plays a pivotal role in the pathogenesis of IR and CVD in diabetes and large clinical trials have demonstrated substantial benefit of the blockade of this system for end-organ protection (Ruilope and Segura 2003; Silverstein et al. 2004; Ball 2003). Indeed, interruption of the RAS with ACEIs or ARBs has been recently shown to prevent the onset of diabetes in hypertensive patients and to reduce cardiovascular and renal disease progression in diabetic patients with hypertension (Ruilope and Segura 2003; Silverstein et al. 2004; Ball 2003).

AII is the major hormone of the RAS that plays an important role in the pathogenesis of hypertension and atherosclerosis (Julius 1990). Several lines of evidence have suggested that AII impairs insulin sensitivity (Rao 1996; Ogihara et al. 2002), and that IR promotes the development of hypertension

by up-regulating the number activity of AII receptors (Nickenig et al. 1998). Studies on hypertensive subjects and animal models have shown improvements in IR in response to treatment with the ARBs (Iimura et al. 1995).

2.4.1 Telmisartan (Micardis®)

Recently, it was reported that telmisartan, a clinically approved ARB with well-documented efficacy in BP reduction, may share some structural homology with the PPARγ ligand, and have capacity to activate PPARγ (Benson et al. 2004; Delea et al. 2003; Mudaliar et al. 2003). In cellular PPARγ transactivation assays, telmisartan was found to produce significant activation of PPARγ when tested at concentrations that achieved in plasma following administration of doses used for the treatment of essential hypertension. By contrast, other commercially available ARBs, namely losartan, eprosartan, candesartan, valsartan, olmesartan, and irbesartan showed little ability to activate PPARγ (Benson et al. 2004; Delea et al. 2003; Mudaliar et al. 2003). In these assays, telmisartan was a moderately potent selective PPARγ agonist, activating the receptor to 25-30% of the maximum level achieved by conventional full agonists, such as pioglitazone and rosiglitazone. Furthermore, the effect was specific for PPARγ, with telmisartan exhibiting weak or no activation of PPARα and PPARβ/δ, respectively when tested at concentrations achieved in plasma with usual oral anti-hypertensive dosing.

It is now believed that the partial agonist behavior displayed by telmisartan may represent potentially important consequences for treatment of MetS. Whereas conventional PPARγ activators, like TZDs, induce full activation of the receptor, selective PPARγ modulators (SPPARMs) (i.e. telmisartan) only induce partial activation. This may result in improvements in glucose and lipid metabolism while causing limited side effects, such as fat accumulation, weight gain, and fluid retention. In animal studies, SPPARMs, unlike conventional PPARγ agonists, have even shown potential to attenuate weight gain (Kurtz and Pravenec 2004).

Telmisartan has also been shown to modulate selectively the expression of key PPARγ target genes when tested at concentrations achievable with therapeutic oral doses. For example, in human visceral adipocytes, telmisartan potently enhanced expression of the PCK1 target gene that encodes phosphoenolpyruvate carboxykinase (PEPCK) (Benson et al. 2004). PEPCK is an enzyme responsible for the increased glyceroneogenesis and FA re-esterification that largely accounts for the ability of PPARγ activators to reduce FA levels (Benson et al. 2004; Tordjam et al. 2003). In addition, recent studies by Fujimoto et al. (2004) have shown that in 3T3-L1 preadipocytes, telmisartan can induce increases in glucose uptake and expression of the glucose transporter 4 (GLUT4).

The anti-diabetic actions of telmisartan associated with its PPARγ-modulating activity have been tested in SD rats fed a high-fat, high-carbohydrate diet over 50 days. Telmisartan administration (~5 mg/kg body

weight/day) caused a significant reduction in serum glucose ($p<0.01$) and TG ($p<0.05$) concentrations. Moreover, the drug significantly attenuated weight gain ($p<0.01$) over 5 weeks (Benson et al. 2004).

The mechanism by which telmisartan is able to activate PPARγ remains to be precisely defined. However, at least part of the answer is likely to lie in its chemical structure, which is quite different from that of the other clinically approved ARBs. Molecular modelling reveals that telmisartan is able to interact with specific amino acid residues in the PPARγ ligand-binding domain and thereby induce receptor activation in a manner similar to that of other partial agonists of PPARγ (Benson et al. 2004). Moreover, given telmisartan's very high volume of distribution, which is much greater than that of the other ARBs, it may have a superior capacity to gain access to the PPARγ–RXR complex within the cell nucleus than the other ARBs (Burnier 2001).

The clinical evidence for telmisartan being a therapeutically important PPARγ activator is ongoing. The data gathered so far strongly suggest that telmisartan treatment can be associated with improvements in glucose and lipid metabolism. For example, in a case study, a 52-year-old male with MetS was started with once-daily telmisartan 80 mg treatment (Pershadsingh and Kurtz 2004). After 8 weeks of treatment, glucose and insulin levels fell to within normal limits. The patient was then switched to once-daily valsartan 160 mg. After 6 weeks of valsartan, his fasting glucose and insulin levels had risen, but returning the patient to telmisartan restored his glucose and insulin levels back

towards normal. This isolated case shows how telmisartan improved the insulin and glucose profile in a high-risk patient with MetS.

3.0 RATIONALE FOR THE STUDY

Although there exists a fairly large body of evidence of research on lifestyle and drug therapy for MetS group of components, there is less information available on diet/drug co-therapy. While the potential metabolic effects of dietary FAs, and pharmacological agents, have been extensively studied both from observational and mechanistic viewpoints, the potential health benefits of dietary CLA, and, more recently, telmisartan in MetS per se, remain underdocumented. Moreover, specifically, research examining CLA benefits in MetS seems to focus on specific groups of genetically modified animals including rats and mice, and there is a paucity of data available on CLA in normal animal models like SD rats, particularly when placed on a high calorie diet that mimic Western lifestyle pattern. Further, research examining therapeutic potentials of CLA when co-administered with pharmacological mediators on MetS is, if any, scarce.

4.0 STUDY HYPOTHESES AND OBJECTIVES

4.1 HYPOTHESES

1. Dietary supplementation with CLA (1.0% w/w) co-administered with telmisartan (5 mg/kg body wt/d) for 20 weeks is safe and well-tolerated by adult SD rats.
2. CLA plus telmisartan co-therapy is an effective way to achieve greater amelioration in body weight and biochemical abnormalities of individual components of MetS in rats fed a high fructose, high lard diet.

4.2 RESEARCH OBJECTIVES

The main purpose of this study was to compare the effects of CLA plus telmisartan treatment with those of each agent alone on several biochemical parameters in a diet-induced MetS rat model.

Specifically, it was of interest to investigate the effects of our treatment protocols on serum lipid profiles, serum glucose and insulin levels, pro-inflammatory and anti-inflammatory cytokine levels, as well as blood pressure, body weight and visceral fat. Simultaneously, the general safety and tolerability of the treatment protocols were investigated.

5.0 MATERIALS AND METHODS

5.1 ANIMALS AND DIETS

The experimental design of the study is summarized in **Figure 2**. Numerous studies (Catena et al. 2003; Oron-Herman et al. 2004, Benson et al. 2004) have suggested SD rat as an appropriate model for diet-induced MetS. In the current study, thirty three, male SD rats – with a mean body weight of 162.5 ± 12.5 g – were obtained at 5 wk of age from the Central Animal Care at the University of Manitoba (Winnipeg, MB, Canada). The presently available data suggest that the pathophysiology of MetS and its contribution to the relative risk of cardiovascular events and heart failure show gender differences, which might be of potential relevance for prevention, diagnostics, and therapy of the syndrome (Regitz-Zagrosek et al. 2006). In recent years, MetS has been more prevalent in men than in women. Because male gender appears to be a risk factor for MetS (Tong et al. 2005), and indeed for CHD (Lunetta et al. 1997; Barrett-Connor 1997), we used male animals.

Rats were housed in stainless steel cages at an ambient temperature of 22-24°C and a 12:12-h light-dark cycle in an environmentally controlled room. For 10 days, the rats were permitted a period of adaptation to the environment, with free access to water and food, at the Animal Facility of St. Boniface Research Centre (Winnipeg, MB, Canada). Thereafter, the rats were randomly assigned to one of five dietary treatments for 20 wk: 1) control (n=7),

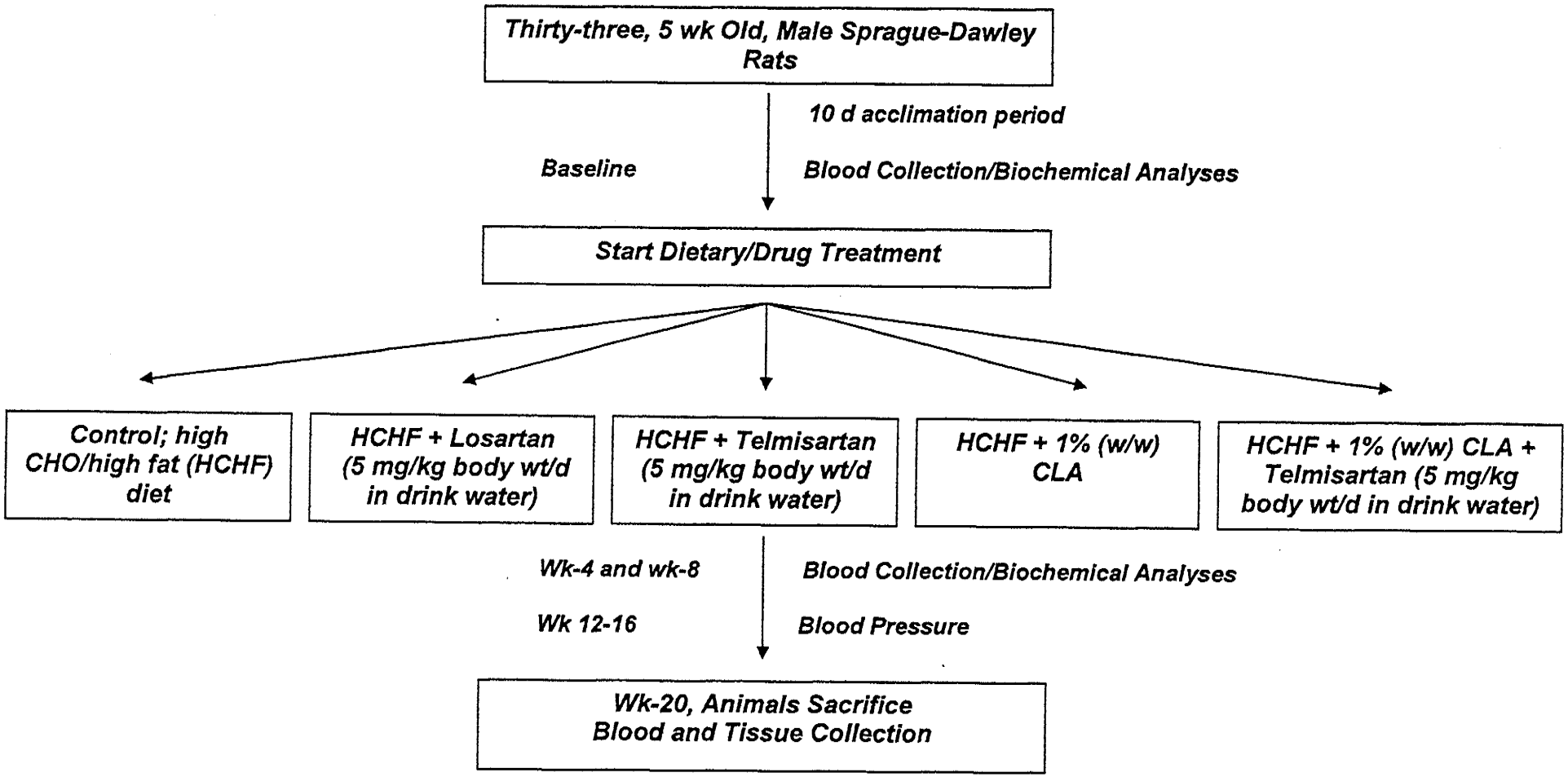


Figure 2. Experimental Design.

2) losartan-treated (n=5), 3) telmisartan-treated (n=7), 4) CLA-treated (n=7), and 5) CLA plus telmisartan-treated (n=7) groups matched with their mean body weights and serum total cholesterol (TC) levels as previously published (Moghadasian et al. 2001; Plat and Mensink 2002; Ostlund et al. 2003). The 5 groups received a diet containing 60% (w/w) fructose and 10% (w/w) lard (TD03293) from Harlan teklad, Wisconsin, USA (**Table 3**) supplemented with 1.0% (w/w) safflower oil (SFO) for each of the control, losartan, and telmisartan groups, or 1.0% (w/w) CLA for each of the CLA and CLA + telmisartan groups daily for the entire period of the study. Both SFO and CLA were stored at -4°C to prevent oxidation. In addition to these diets, telmisartan (Micardis®) and losartan (Cozaar®) were dissolved in water and provided to the appropriate treatment groups of animals daily. Diet/oil and drug treatment protocols are summarized in **Table 4.** According to manufactures' information (Boehringer Ingelheim 2004 and E.I. du Pont de Nemours and Company 2003) and previous studies (Benson et al. 2004), both drugs are stable to heat (15-30°C). The experimental groups received the high caorie diet for 20 wk; an appropriate period to induce the MetS (Catena et al. 2003; Oron-Herman et al. 2004). During the experimental course, animals' body weights were recorded weekly and food and water intakes were measured each day.

At baseline, wk 4, and wk 8, blood samples were obtained in the non-fed state by jugular venipuncture. The night before blood sampling, food was removed from animals' cages at 8:30 PM, and blood was drawn the following morning starting from 8:30 AM. Blood samples were taken from the jugular

Table 3. Composition of the High-Carbohydrate, High-Fat Diet (TD03293 by Harlan teklad).

Ingredient	g/kg diet	Percentage
Casein	207.0	20.7%
DL-Methionine	3.0	0.3%
Fructose	600.0	60%
Lard	100.0	10%
Cellulose	42.0	4.2%
Calcium Carbonate	3.0	0.3%
Mineral Mix, AIN-76	35.0	3.5%
Vitamin Mix, Teklad	10.0	1.0%
Total	**1000**	**100%**

Table 4. Summary of the Experimental Groups and their Diet/Drug Treatment Protocols.

Experimental Groups	Diet/Drug Protocols
Control (n=7)	Control diet (high-carbohydrate, high-fat) + 1.0% (w/w) safflower Oil
Losartan-treated (n=5)	Control diet/safflower oil + 5 mg/kg body weight/day losartan in drinking water
Telmisartan-treated (n=7)	Control diet/safflower oil + 5 mg/kg body weight/day telmisartan in drinking water
CLA-treated (n=7)	Control diet + 1.0% (w/w) CLA
CLA+telmisartan-treated (n=7)	Control diet + 1.0% (w/w) CLA + 5 mg/kg body weight/day telmisartan in drinking water

High-carbohydrate, high-fat= 60% (w/w) fructose, 10% (w/w) lard. CLA, conjugated linoleic acid of 75% mixture of c9,t11 and t10,c12 in a 50/50 ratio.

vein of animals under light anesthesia (induced by 1-2% isoflurane) and placed into serum separator tubes (Tyco Healthcare Group LP, Mansfield, MA, USA). Following collection, blood samples were allowed to clot at room temperature for 15 minutes before they were put on ice, and sera were separated from blood by centrifugation at 4°C (7000 rpm for 10-15 minutes) and were used for biochemical analyses. Final blood sample (wk 20) was collected through cardiac puncture in test tubes with 15% EDTA (Tyco Healthcare Group LP, Mansfield, MA, USA) after euthanization by CO_2. On the morning of the last day of study, each rat was injected intraperitoneally (ip) with 400 µg lipopolysaccharide (LPS) and 100 i.u of heparin, 2 hours and 10 minutes before sacrifice, respectively. Plasma was separated from blood samples by centrifugation at 4°C (7000 rpm for 10-15 minutes).

All animals looked healthy and active during the experimental period. Hearts, aortas, livers, kidneys, spleens, and peritoneal adipose tissues were collected at the end of the study and fixed and/or frozen at -80°C for histological and/or molecular examinations in future studies.

5.2 DIETARY OILS AND MEDICATIONS

Based on the limited reports available, it is possible to suggest that preparations enriched mainly with c9,t11 and t10,c12 CLA isomers are preferable for human consumption compared to preparations containing more isomers, in terms both of safety and efficacy (Gaullier et al. 2002). Therefore, in our study, CLA was composed of a 75% mixture of the two main isomers

c9,t11 and t10,c12 in a 50:50 ratio (34.0% of c9,t11 and 34.1% of t10,c12) and 8.1% of other miscellaneous isomers. The use of this mixed isomer form of CLA is most relevant regarding the impact of CLA on the management of T2DM as this source of CLA is currently available commercially for use in humans (e.g. Tonalin™; Kelley et al. 2001). For purposes of this study, CLA was provided in-kind by Bioriginal Science and Food Corp. (SK, Canada). SFO, being a good source of omega-6, but not omega-3, FAs was obtained from Dyets Inc. (PA, USA), and included in the diet of groups that did not receive CLA to avoid essential FA deficiency.

The AII receptor antagonists, telmisartan (Micardis®; Boehringer Ingelheim) and losartan (Cozaar®; E.I. du Pont de Nemours and Company) were purchased from St. Boniface General Hospital Pharmacy, Winnipeg, MB, Canada, and administered by dissolving the commercially available medications in the drinking water (dose ~5 mg/kg body weight/day). Unlike telmisartan, losartan is not a partial agonist of PPARγ and was, thus, incorporated in the study as a "control" drug for telmisartan as described in a previous study (Benson et al. 2004).

5.3 DATA COLLECTION

5.3.1 Lipid Analysis

5.3.1.1 Total Cholesterol

Serum TC levels were measured using a standard enzymatic kit (Diagnostic Chemicals Limited, Charlottetown, PE, Canada). Solutions with known concentrations of cholesterol (Roche company) were used to generate a standard curve with an R^2 value >0.995 that was subsequently used to calculate the cholesterol concentrations in the experimental samples (Moghadasian et al. 1997).

The procedure involved a number of steps: cholesterol esters were enzymatically hydrolyzed by cholesterol esterase (present in the reagent supplied) to form cholesterol and FFAs; free cholesterol was then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide; hydrogen peroxide combined with hydrobenzoic acid and 4-aminoantipyrine in the presence of peroxidase to form the chromophore, which was quantified at 500-550 nm; after incubation absorbance was read using a spectrophotometer (Perkins-Elmer company). Cholesterol concentrations were calculated considering the dilution factor. Quality control assurance was established using "control samples" from the manufacturer (Roche company). All experimental samples were analyzed in duplicate and mean values were calculated.

5.3.1.2 Triglycerides

Serum TG concentrations were quantified using a standard enzymatic kit (Diagnostic Chemicals Limited, Charlottetown, PE, Canada; Moghadasian et al. 1999). A standards solution (200 mg/dL) provided by the manufacture was used to produce a linear response curve. Five μL of samples plus 300 μL of color reagent were added to each well and the plate was incubated at room temperature for 10 minutes. During this time, TG were hydrolyzed by LPL to glycerol and FFAs. The glycerol was phosphorylated in the presence of ATP and glycerolkinase to form glycerol-1-phosphate and ADP. Glycerol-1-phosphate was then oxidized by glycerophosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide and 4-aminoantipyrine and N-ethyl-N(3-sulphopropyl)m-anisidine were catalyzed by peroxidase to form a quinoneimine dye that could be quantified at 540 nm. Quality control assurance was established using "control samples" from the manufacture (Roche company). All experimental samples were analyzed in duplicate and mean values were calculated.

5.3.1.3 HDL-cholesterol

Serum HDL-cholesterol levels were assessed using a standard precipitation method (Moghadasian et al. 2001), based on precipitation of VLDL and LDL particles followed by quantification of HDL-cholesterol in the supernatant. Standards were made using standard solution (200 mg/dL of cholesterol) provided by the manufacture (Diagnostic Chemicals Limited,

Charlottetown, PE, Canada) to produce a linear response curve and the "control samples" were prepared using materials from Roche Company. Into each tube 20 μL of the samples plus 50 μL of HDL precipitation reagent were placed, mixed vigorously, and incubated for 10 minutes at room temperature. After 10 minutes, tubes were centrifuged at 2666*g* (7000 rpm, Ependorf centrifuge 5804 R) at 4°C for 30 minutes. Thirty μL of the supernatant plus 250 μL of color reagent were added and the plate was incubated at room temperature for 10 minutes. The cholesterol values in supernatants were measured using a standard kit (Diagnostic Chemicals Limited, Charlottetown, PE, Canada) as described above. Quality control assurance was established using "control samples" from the manufacturer (Roche Company). All experimental samples were analyzed in duplicate and mean values were calculated. The lipid measurements in "control solutions" always showed values fit within actual values reported by the manufacturer.

5.3.1.4 LDL-cholesterol

Serum LDL-cholesterol levels were calculated according to Friedewald formula: $C_{LDL} = C_{plasma} - C_{HDL} - TG/2.2$ (Friedewald et al. 1972). This method for estimating plasma LDL-cholesterol concentrations provides a reasonable approximation that is useful for many purposes (Johnson et al. 1997; Gazi et al. 2006).

5.3.2 Glucose Analysis

Serum glucose levels were determined using a commercially available glucose-oxidase kit (Wako Chemicals Inc., Richmond, VA, USA; Stark et al. 2000). Standard solutions with known concentrations of glucose (Wako Chemicals Inc.) were used to generate standard curve with an R^2 value >0.990. Accordingly, glucose concentrations in the experimental samples were calculated.

It has been demonstrated that β-glucose oxidase promotes oxidation of glucose by molecular oxygen to gluconic acid. Hydrogen peroxidase is simultaneously produced in this reaction. Enzymatic methods for the determination of glucose have been known to combine glucose oxidase (GOD), peroxidase (POD), and an oxygen acceptor (chromogen) that has high specificity and simplicity. The equilibrium of D-glucose in standard solutions is usually maintained in the ratio of α-D-glucose 36.5% and β-D-glucose 63.5%. GOD reacts only with β-D-glucose.

In our glucose assay, each test tube was accurately loaded with 20 μL of serum samples or standard solutions and 3.0 mL of working solution, mixed vigorously, and incubated for 5 minutes in a water bath at 37°C. When a test sample was allowed to react with the reagent, α-D-glucose present in the sample was converted rapidly to the β-isomer by the action of mutarotase, and was then oxidized by GOD to produce hydrogen peroxide. The produced hydrogen peroxide induced oxidative condensation between phenol and 4-aminoantipyrine in the presence of POD, so that a red color was produced.

The amount of glucose contained in the test sample was then determined by measuring the absorbance of the red color at 505 nm using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, Cambridge, United Kingdom). All experimental samples were analyzed in duplicate and mean values were calculated.

5.3.3 Blood Pressure

Blood pressure (BP) was recorded at wk 12 of the study using the standard tail-cuff method in conscious animals as previously reported (Donnelly et al. 1995; Richey et al. 1998). This method is widely used to record blood pressure of animals in a calm/safe environment for accurate and reproducible data. Rats were assigned to training sessions every day for 3-4 days before the blood pressure was measured.

For each rat (done one at a time), the following components were used: 1) a tail cuff; 2) an inflation device and pressure readout; 3) a pulse sensor (highly sensitive pressure transducer which detects pulsations in a small rubber bulb; the bulb is taped to tail distal to the cuff); 4) a readout for the pulse sensor, MP100WS Workstation, which includes acquisition hardware, AcqKnowledge software, cables, and manuals; 5) one amplifier for the pulse transducer and one amplifier to record pressure; and 6) a low-cost disposable pressure transducer.

BP was recorded in rat tails in a manner similar to that used to measure BP non-invasively in humans. A cuff was placed around the tail, and inflated

above the systolic pressure. This caused pulsations at a more distal pulse sensor to cease. As the cuff was slowly deflated, the reappearance of pulsations was noted, and the cuff pressure at this time was recorded as the systolic pressure (mmHg) in the tail.

5.3.4 Oral Glucose Tolerance Test

At the end of the study (wk 20), glucose tolerance testing (oral glucose tolerance test; OGTT) was performed in conscious animals as previously reported (Benson et al. 2004). Oral administration of glucose (100 mg/100 g body weight, dissolved in water) by gastric gavage was performed in overnight-fasted animals. Glucose stock solution was prepared by dissolving 7 g of D-(+)-GLUCOSE (Sigma-Aldrich, Oakville, ON, Canada) in 20 ml of distilled water. Blood glucose levels were assessed in collected tail blood, and glucose level data were recorded immediately before and 30, 60, and 120 minutes after glucose administration by One Touch Ultra Blood Glucose Meter (LifeScan Inc., Milpitas, CA, USA; Schlenker et al. 2004).

For glucose measurement, the following steps were taken: 1) the test strip was inserted into the glucometer (meter turned on automatically and a symbol was flashed with the unit of measurement), 2) a drop of blood was applied to a narrow channel in top edge of the test strip, 3) the blood drop was held to the top edge of the test strip until a confirmation window was completely filled, before the meter began to count down, and 4) blood glucose test readings were displayed after the meter counted from 5 to 1.

5.3.5 Insulin

At wk 20, plasma insulin levels were quantified using the Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL, USA; Bhattacharya et al. 2005). Before assay procedures took place, Rat Insulin Standard (Lyophilized; 2.56 ng/vial), provided by the manufacturer, was reconstituted with the addition of 100 μl distilled water to provide a Stock Solution of 25.6 ng/mL. As well, Working Rat Insulin Standards were prepared by labeling eight microtubes 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 ng/mL, and dispensing 150 μl and 50 μl of Sample Diluent (by the manufacturer) into the 6.4 ng/mL tube and each of the other tubes, respectively. Then, 50 μl of the Rat Insulin Standard Stock Solution was pipetted into the 6.4 ng/mL tube, and mixed well. Similarly, 50 μl of 6.4 ng/mL standard was dispensed into the 3.2 ng/mL and mixed thoroughly. The dilution procedure was repeated on the remaining tubes, except for the 0 standard, which only contained Sample Diluent of 50 μl.

In this assay, the following steps were taken: 1) after modules were placed in the micoplate, 5 μl of the plasma sample (or Insulin Standards) were pipetted to each well, and plate was covered with a plastic microplate cover, and incubated for 2 h at 4°C. During this reaction, rat insulin in the sample bound to the guinea pig anti-rat insulin antibody coated on the microplate wells, 2) the well's contents were aspirated and washing buffer was used to wash the wells 5 times (300 μl per wash); unbound materials were removed by washing, 3) 100 μl of Anti-Rat Insulin Enzyme Conjugate (prepared by diluting

one bottle of Anti-Rat Insulin Enzyme Conjugate Stock Solution with one bottle of Enzyme Conjugate Diluent) was pipetted to each well, 4) the plate was covered and left for 30 min. at room temperature. During this step, horseradish peroxidase (POD)-conjugated anti-rat insulin antibody bound to the guinea pig anti-rat insulin, and the antibody/rat insulin of the complex was immobilized on the microplate well, 5) the contents of the wells were discarded and the wells were washed 7 times (300 μl per wash) using buffer. Excess POD-conjugate was removed by washing, 6) immediately after washing, 100 μl of Enzyme Substrate Solution (provided by the manufacturer) was pipetted, and left for 40 min. at room temperature in dark, 7) the reaction was stopped by the addition of 100 μl of Enzyme Reaction Stopping Solution (from the manufacturer). At this step, the bound POD conjugate on the microplate well was detected with 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution, and 8) the absorbance of the solution was measured within 30 minutes at 450 nm and 620 nm (subtracting) wavelength. All experimental samples were analyzed in duplicate and mean values were calculated.

Rat insulin concentration was determined by the standard curve obtained by plotting the absorbance versus corresponding concentration of rat insulin standard.

5.3.6 Cytokines

At wk 20, animals were injected with 400 mg LPS from *Escherichia coli* ip 2 hours before sacrifice. Usually, in non-stimulated state, the levels of

cytokines in blood are below the detection limit; LPS (Sigma-Aldrich, Oakville, ON, Canada) was injected to stimulate the production of multiple cytokines. Final blood samples were collected through cardiac puncture and centrifuged, and plasma was obtained. For this analysis, plasma samples of each experimental group were pooled into a new test tube, and assayed with the RayBio™ Rat Cytokine Array system I & 1.1 map (RayBiotech Inc., Norcross, GA, USA).

Rat cytokine array membranes coated with 19 specific cytokine antibodies were probed with isolated protein samples. The membranes were blocked by incubation with the blocking buffer at room temperature for 30 min and incubated with sample at room temperature for 1 h. Membranes were washed three times with Wash Buffer I and two times with Wash Buffer II at room temperature for 5 min per wash and incubated with biotin-conjugated antibodies at 4 °C overnight. Finally, the membranes were washed, incubated with HRP-conjugated streptavidin at room temperature for 1 h and with detection buffer for 1 min, and exposed to X-ray film for 40 s (Kodak, Inc.). The exposed films were digitized and the relative cytokine levels were compared after densitometry analysis (Scion NIH Image 1.63). The relative protein levels were obtained by subtracting the background staining and normalizing to the positive controls on the same membrane. Probing with buffer that was devoid of protein samples did not produce positive staining except at the positive control spots coated with the biotinylated IgGs. The RayBio™ cytokine array identified profiles of multiple cytokines in plasma samples as previously

reported (Watanabe et al. 2005). Cytokine concentrations were expressed in optical density.

5.4 ETHICS

Animal care and all experimental procedures of this study received ethics approval from the Animal Care Committee on the use of Animals in Research, at the University of Manitoba, Winnipeg, MB, Canada.

5.5 SAMPLE SIZE AND STATISTICAL ANALYSIS

Data were analyzed using SPSS 11.5 statistical software for Windows (SPSS Inc., Chicago, IL, USA). Results were expressed as mean ± one standard deviation (SD) unless otherwise stated. The level of statistical significance was set at $p<0.05$. One-way analysis of variance (ANOVA) was selected to determine differences in outcome measurements between dietary treatment groups. Post-hoc analysis was performed using the Tukey multiple group comparison procedure to determine significant differences among various experimental groups.

An estimated sample size of n = 5-7 per diet group was based on previous studies with a sufficient statistical power of >90% for detecting difference of ½ SD as significant at the level of $p<0.05$. This applies to both biochemical parameters and the morphometrical measurements.

6.0 RESULTS

6.1 BODY WEIGHT

Initial body weights of rats (336.4 ± 25 g) were not significantly different among the experimental groups. **Figure 3** demonstrates that rats in all the experimental groups gained weight during the 20-wk study. However, the extent of mean weight gain was significantly lower in the CLA + telmisartan-treated rats relative to the control, CLA-, and losartan-treated groups.

By wk 4 of the study, all treated animals exhibited slightly lower mean body weights when compared to controls. Of these, specifically, the group treated with CLA + telmisartan showed the lowest body weight with no statistically significant differences.

Throughout the course of the study, all groups continued to gain weight as expected. None of the losartan-, telmisartan-, and CLA-treated groups showed any significant attenuation in weight gain. Here, CLA and losartan observations agree with previous findings in same animal model (Yamasaki et al. 2003; Choi et al. 2004) and (Benson et al. 2004), respectively. Telmisartan-treated rats, on the other hand, showed a trend toward reduction in body weight starting from wk 8 and till the end of the study as compared to control animals.

As of wk 8, CLA + telmisartan-treated rats started to show a significantly lower mean body weight relative to the control ($p<0.01$) and CLA-treated ($p<0.05$), but not other, groups. This significant attenuation in weight gain was observed in the combination group throughout the study period,

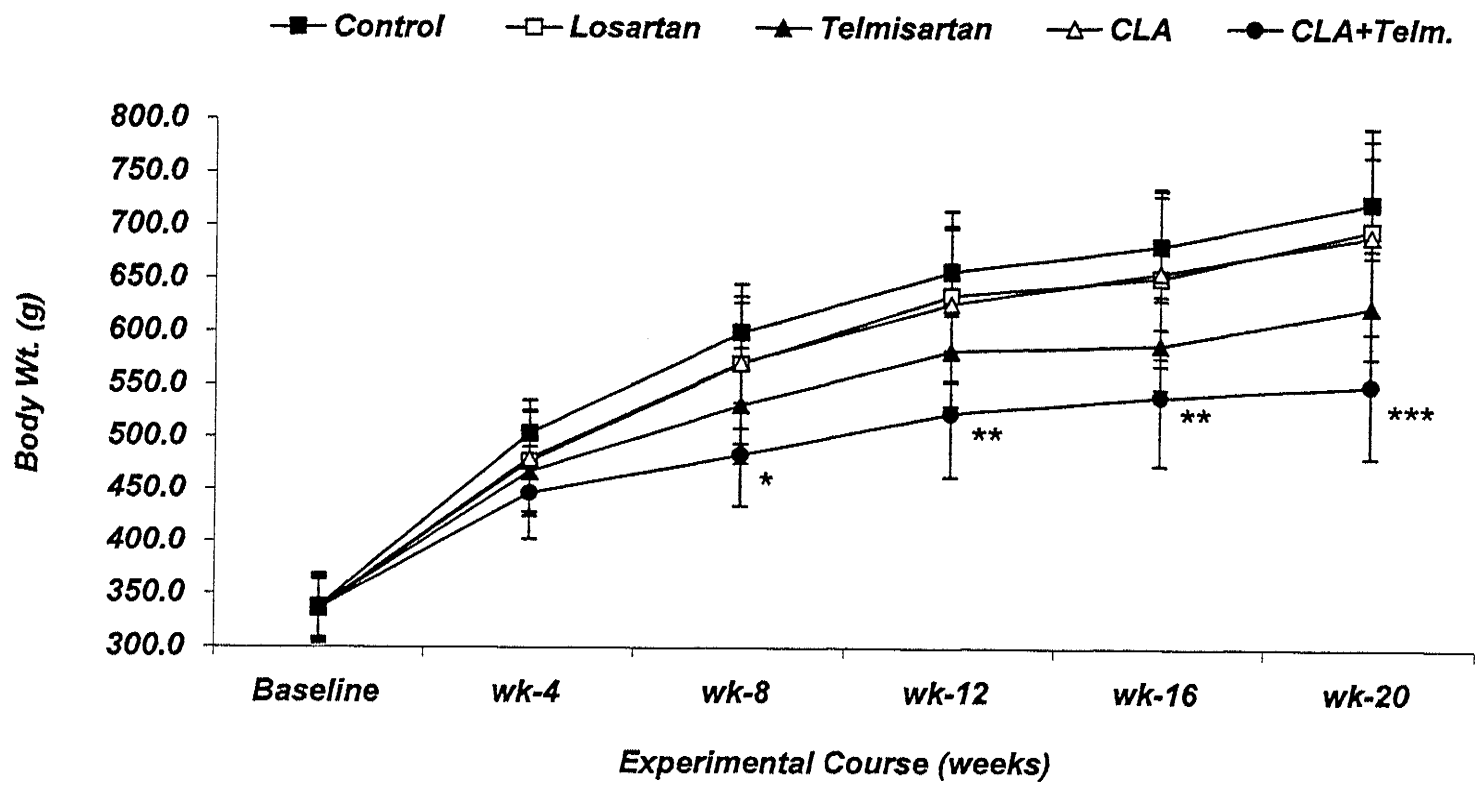


Figure 3. Effects of dietary protocols on body weight at baseline and 4 wk intervals. *Values are means ± SD, n= 7 except losartan-treated group (n= 5). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water. * indicates significant difference at p<0.01 vs. control and p<0.05 vs. CLA, ** indicates significant difference at p<0.01 vs. control and p<0.05 vs. losartan, *** indicates significant difference at p<0.01 vs. control, CLA, and losartan.*

however at different extents (26%, 26%, and 31% less for wk 12, wk 16, and wk 20, respectively; $p<0.01$) as compared to the controls. As well, the co-therapy resulted in significantly lower mean body weight when compared to the losartan-treated ($p<0.05$) animals at wk 12 and wk 16.

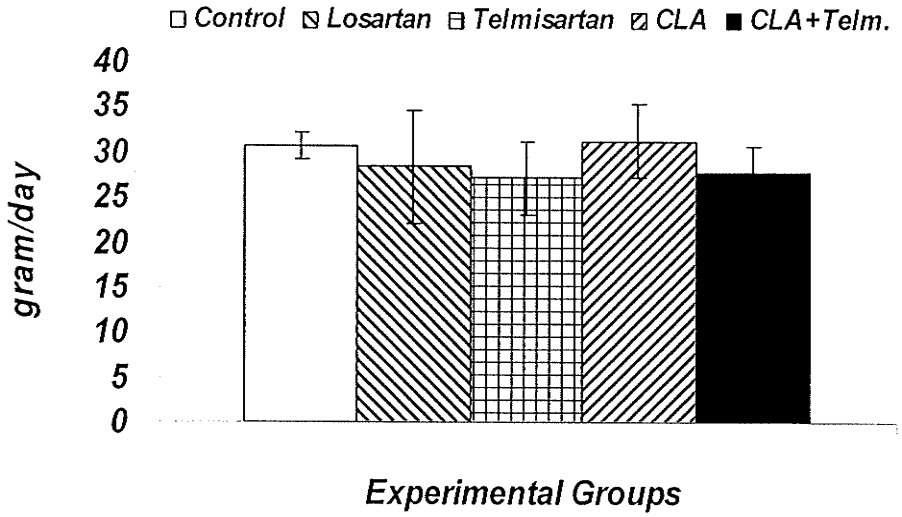
By wk 20, CLA- and telmisartan-treated rats exhibited 4% and 16% decrease in mean body weight, respectively, relative to the controls. When CLA was co-administered with telmisartan, a 31% reduction in mean body weight was observed in comparison to the control group. As well, CLA/telmisartan co-administration resulted in significantly lower body wt, at wk 20, compared to the losartan- or CLA-treated animals ($p<0.01$). Based on these observations, the co-administration of CLA and telmisartan appears to have an additive effect on body weight reduction in SD rats.

6.2 FOOD AND WATER INTAKES

To ensure the delivery of correct drug doses, the drug concentrations were adjusted in the drinking water each week based on the average water consumption and body weights in each group. As well, we measured daily food intake for each rat by subtracting the amount of food remaining in the cage from the measured amount of food provided each day. The average daily food intake for each rat was then calculated by averaging all of the daily intake measurements obtained over the entire course of the study.

Food and water intakes are shown in **Figure 4-A** and **4-B,** respectively. Average daily food intakes were similar (29 ± 3.6 g/d) among the different

A.



B.

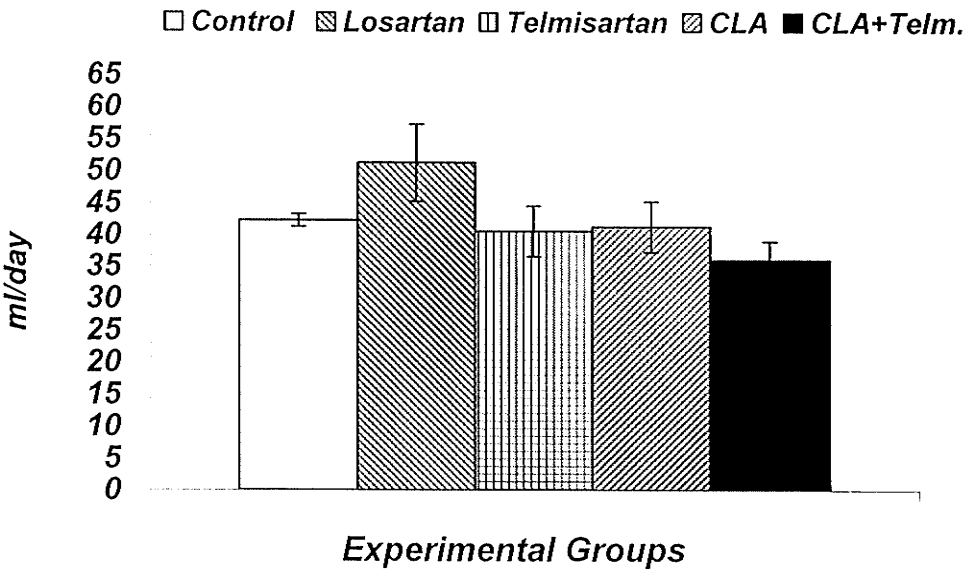


Figure 4. Effects of dietary protocols on daily food (A) and water (B) intakes. *Values are means ± SD, n= 7 except losartan-treated group (n= 5). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water.*

experimental groups. Thus, the attenuation of weight gain induced by the CLA + telmisartan protocol could not be attributed to reduced energy intake. Similarly, average water intakes (42.3 ± 5.4 ml/d) were nearly equal among all 5 groups.

6.3 SERUM LIPID PROFILES

6.3.1 Total Cholesterol Concentrations

Levels of serum TC at baseline and during the experimental course are illustrated in **Figure 5.** At wk 4 of the study, serum TC levels were slightly decreased in the telmisartan-, CLA-, and the CLA + telmisartan-treated groups as compared to the control and losartan-treated rats.

At wk 8, the CLA/telmisartan-treated rats exhibited a significant ($p<0.05$) reduction of approximately 48% in serum TC levels relative to the control or losartan-treated rats. None of the other treatments had such effects. Yet, both telmisartan- and CLA-treated rats did show reductions in levels of TC (17% and 15% less, respectively) with no significant differences as compared to the controls and losartan-treated animals. As in body weight, it seems that the co-administration of CLA and telmisartan has an additive effect on reducing serum TC levels.

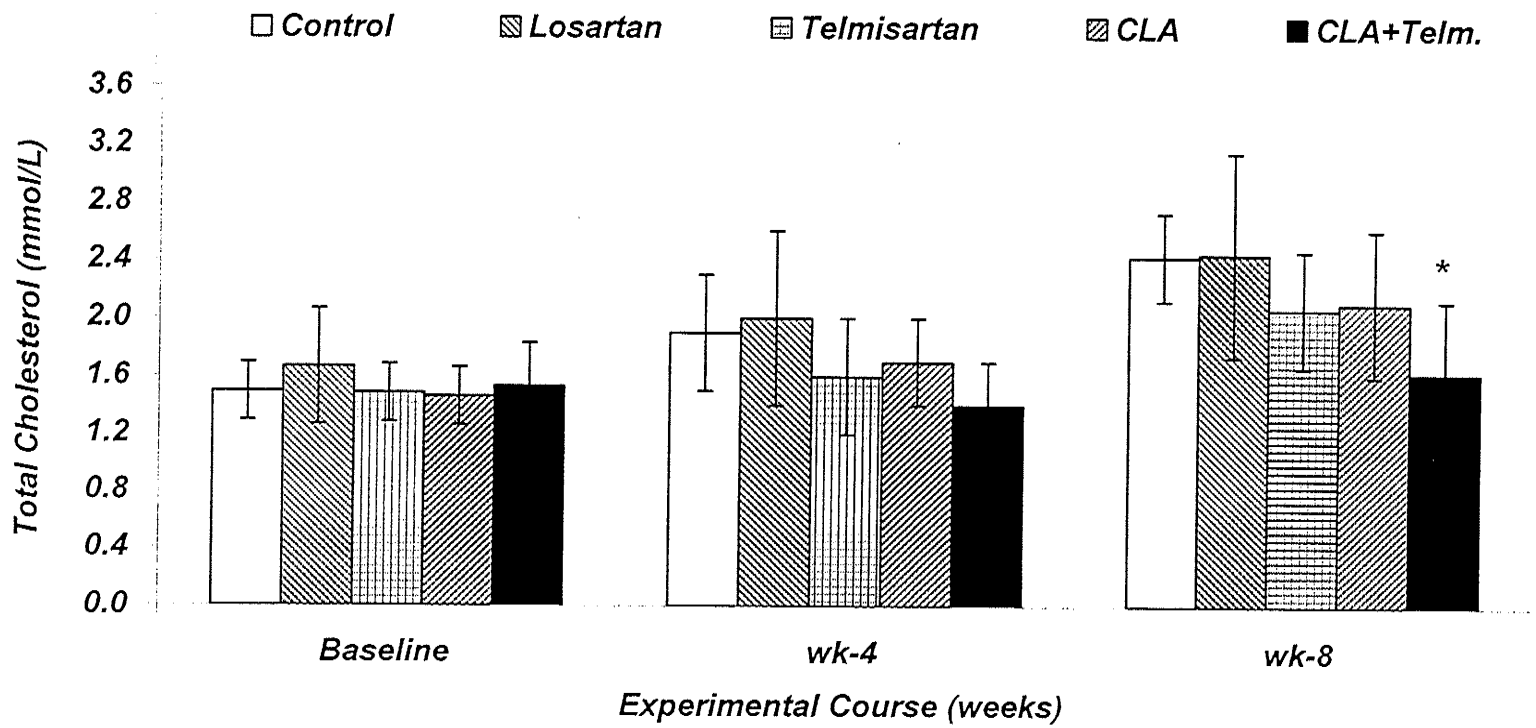


Figure 5. Effects of dietary protocols on serum total cholesterol (TC) concentrations. *Values are means ± SD, n= 7 except losartan-treated group (n= 5). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water. * indicates significant difference at p<0.05 vs. control and losartan.*

6.3.2 Triglyceride Concentrations

Figure 6 illustrates concentrations of serum TG in all of the experimental groups at baseline, wk 4, and wk 8 of the study. As is evident and in agreement with previous studies (Benson et al. 2004; Catena et al. 2003; Ackerman et al. 2005), the "high-fructose" diet increased serum TG levels of all groups in as early as 4 weeks on the dietary protocols. Serum TG concentrations were, however, significantly reduced ($p<0.01$) in the CLA/telmisartan-treated animals as compared to controls (1.0 ± 0.5 vs. 2.3 ± 0.6 mmol/L, respectively). Other treated-groups showed slight reductions in serum TG levels relative to the control group.

At wk 8, serum TG concentrations in the control, losartan-, and CLA-treated rats were higher than those in the telmisartan- and CLA/telmisartan-treated groups (3.8 ± 0.7, 3.0 ± 1.8, and 2.9 ± 1.2 vs. 2.0 ± 1.1 and 1.1 ± 0.2 mmol/L, respectively). In a previous study by Benson et al. (2004), pharmacological dose of 5 mg/kg body weight/day of telmisartan, but not losartan, significantly reduced serum TG levels in SD rats over 5 weeks. We observed similar effects. Telmisartan, but not losartan, significantly attenuated the increase in serum TG levels (~90%; $p<0.05$) as compared to controls at wk 8 on the high-calorie dietary protocol. CLA alone resulted in a 31% non-significant reduction. When combined, CLA and telmisartan resulted in more significant reduction in serum TG concentrations after 8 weeks on the high-carbohydrate, high-fat diet as compared to controls ($p<0.001$), and losartan- and CLA-treated ($p<0.05$) animals.

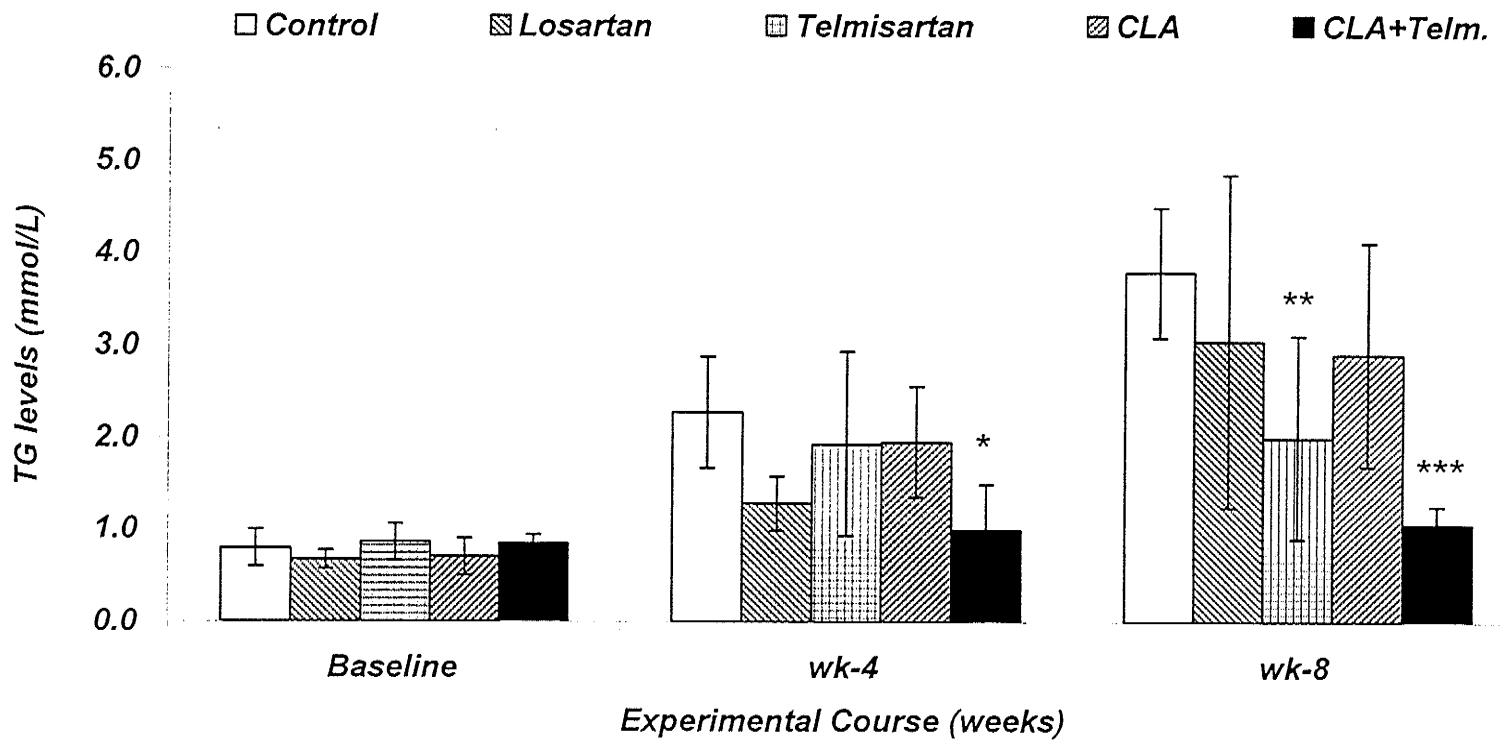


Figure 6. Effects of dietary protocols on serum triglyceride (TG) concentrations. *Values are means ± SD, n= 7 except losartan-treated group (n= 5). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water. * indicates significant difference at p<0.01 vs. control, ** indicates significant difference at p<0.05 vs. control, *** indicates significant difference at p<0.001 vs. control and p<0.05 vs. losartan and CLA.*

6.3.3 HDL-cholesterol Concentrations

Figure 7 shows the levels of serum HDL-cholesterol in the experimental groups. As seen with TC at wk 4 of the study, serum HDL-cholesterol levels were slightly higher in control- and losartan-treated rats relative to other groups. For example, compared to CLA/telmisartan group, rats in the control group had a non-significant 17% more serum HDL-cholesterol levels.

Similar to wk 4, no significant differences were observed at wk 8 of the study. CLA-, telmisartan-, and CLA + telmisartan-treated animals showed slight reductions in HDL-cholesterol levels (0.52 ± 0.14 mmol/L and 0.52 ± 0.21 mmol/L for CLA and telmisartan, respectively, and 0.47 ± 0.06 mmol/L for CLA/telmisartan) as compared to controls (0.60 ± 0.08 mmol/L). Whereas co-administration of CLA and telmisartan resulted in a 28% reduction in HDL-cholesterol, each of CLA alone and telmisartan alone caused only 15% less serum HDL-cholesterol. In contrast, losartan-treated rats had almost identical mean levels of serum HDL-cholesterol to those of control group (0.62 ± 0.13 vs. 0.60 ± 0.08 mmol/L, respectively).

The higher serum HDL-cholesterol levels in control and losartan-treated animals might be a normal consequent as TC was also higher in these two groups compared to others after 8 weeks on the dietary protocol.

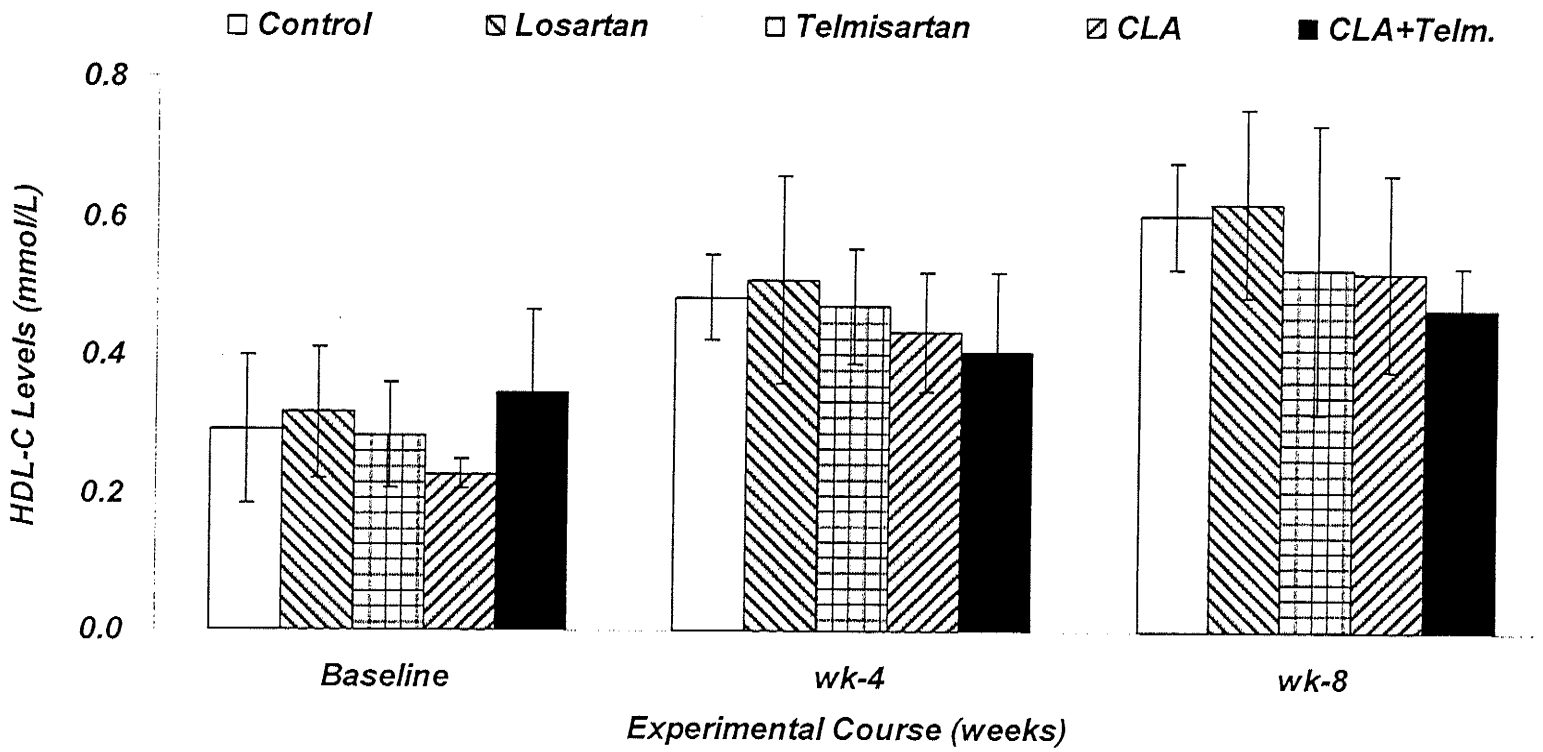


Figure 7. Effects of dietary protocols on serum HDL-cholesterol concentrations. *Values are means ± SD, n= 7 except losartan-treated group (n= 5). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water.*

6.3.4 LDL-cholesterol Concentrations

Levels of serum LDL-cholesterol in all experimental groups are illustrated in **Figure 8.** LDL-cholesterol concentrations, calculated by the Friedewald equation (TC-HDL-TG/2.2), were not significantly different among treatment protocols throughout the study period. For example, even though LDL-cholesterol levels were slightly higher in control- and losartan-treated animals after 4 weeks on feeding, no statistically significant differences were observed.

Of note is that the CLA/telmisartan-treated rats had non-significant 22% less LDL-cholesterol in serum at wk 4, and 13% less LDL-cholesterol at wk 8 of the study as compared to controls.

6.4 SERUM GLUCOSE LEVELS

Figure 9 exhibits serum glucose levels in all of the 5 experimental groups. Serum glucose concentrations in the control group over 8 weeks of the study period looked comparable (8.0 ± 1.0 vs. 8.9 ± 0.7 vs. 8.6 ± 0.6 mmol/L for baseline, wk 4, and wk 8, respectively).

At wk 4 of the study, losartan-treated animals showed significant reduction in serum glucose levels (~39%; $p<0.05$) as compared to CLA group. When compared to controls, glucose concentrations were ~27% less in losartan, ~16% less in telmisartan, and 11% less in the combination groups, but ~9% more in CLA group with no significant differences. The effect of telmisartan observed here is similar to that reported by Benson et al. (2004) in

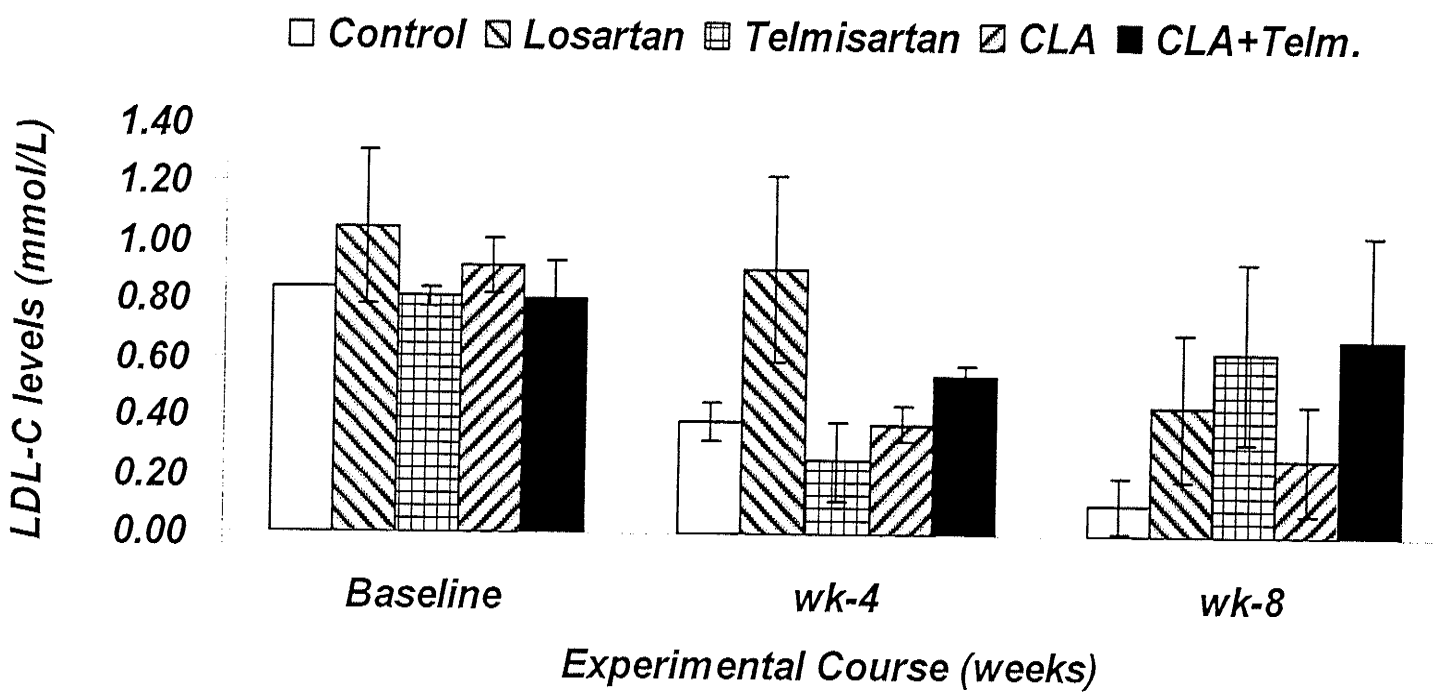


Figure 8. Effects of dietary protocols on serum LDL-cholesterol concentrations. *Values are means ± SD, n= 7 except losartan-treated group (n= 5). LDL-cholesterol levels calculated using Friedewald equation (formula: TC-HDL-TG/2.2). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water.*

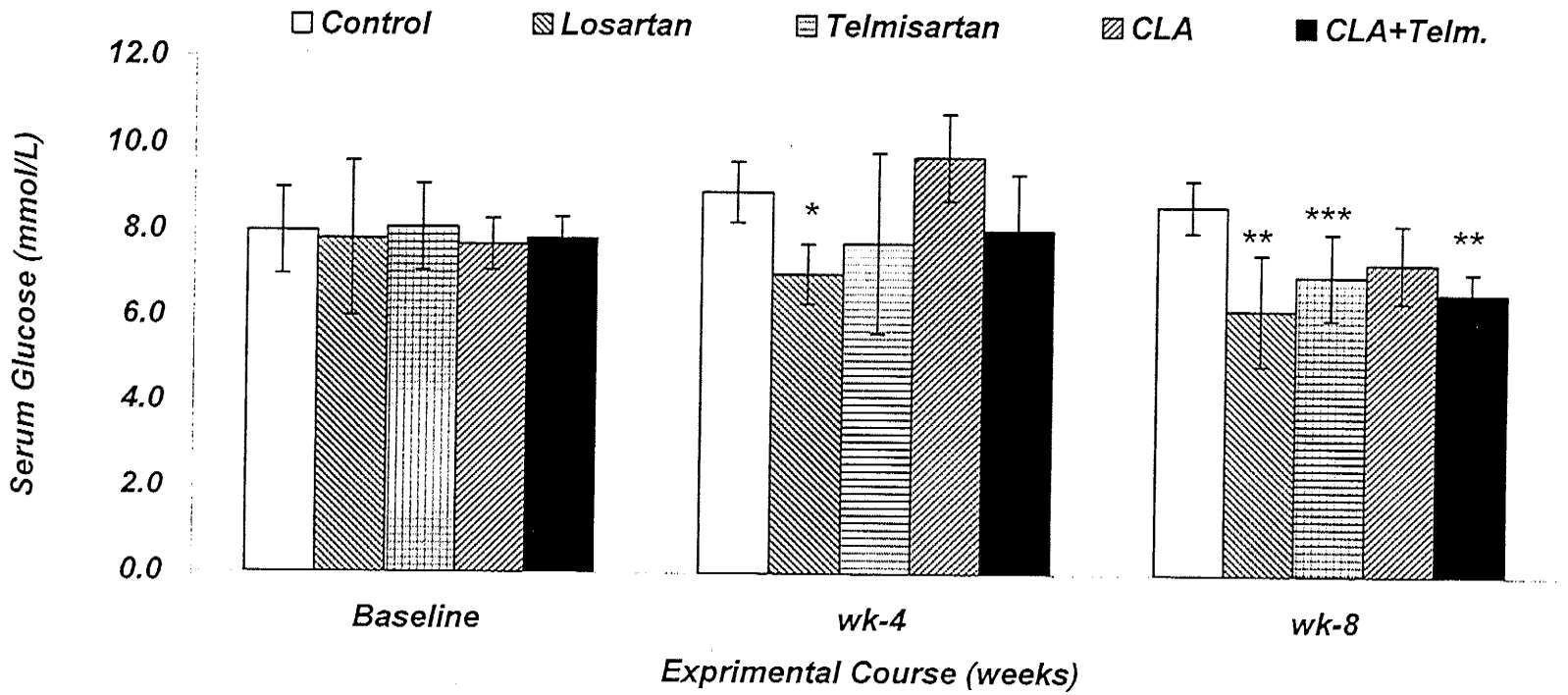


Figure 9. Effects of dietary protocols on serum glucose concentrations. *Values are means ± SD, n= 7 except losartan-treated group (n= 5). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water. * indicates significant difference at p<0.05 vs. CLA, ** indicates significant difference at p<0.01 vs. control, *** indicates significant difference at p<0.05 vs. control.*

which administration of telmisartan, in 5 weeks on a high calorie diet, resulted in ~12% less serum glucose levels as compared to controls.

At wk 8, both losartan- and CLA/telmisartan-treated animals showed significantly lower serum glucose levels (39% and 30%, respectively; $p<0.01$) relative to their counterpart controls. Telmisartan-treated animals exhibited statistically-significant 23% less serum glucose levels ($p<0.05$) as compared to controls whereas no significant differences were observed between CLA-treated and control rats.

6.5 SYSTOLIC BLOOD PRESSURE

Figure 10 shows data of SBP in experimental groups. For the measurements, 5 readings were obtained from each rat and averaged, after the highest and the lowest values were excluded as previously cited (Nagao et al. 2003a).

At wk 12, the control group exhibited a notably higher SBP in comparison to other treatment protocols. Administration of the two anti-hypertensive drugs caused significant reduction in SBP as compared to controls and other groups however, to different extents. Losartan-treated animals exhibited significant reduction in SBP as compared to controls (113.0 ± 4.0 mmHg vs. 138.0 ± 8.0 mmHg; $p<0.001$). Telmisartan-treated rats showed similar reduction as compared to controls (103.0 ± 7.0 mmHg vs. 138.0 ± 8.0 mmHg; $p<0.001$) and CLA-treated (103.0 ± 7.0 mmHg vs. 128.0 ± 10.0 mmHg; $p<0.001$) rats, and a lesser reduction in SBP relative to the CLA +

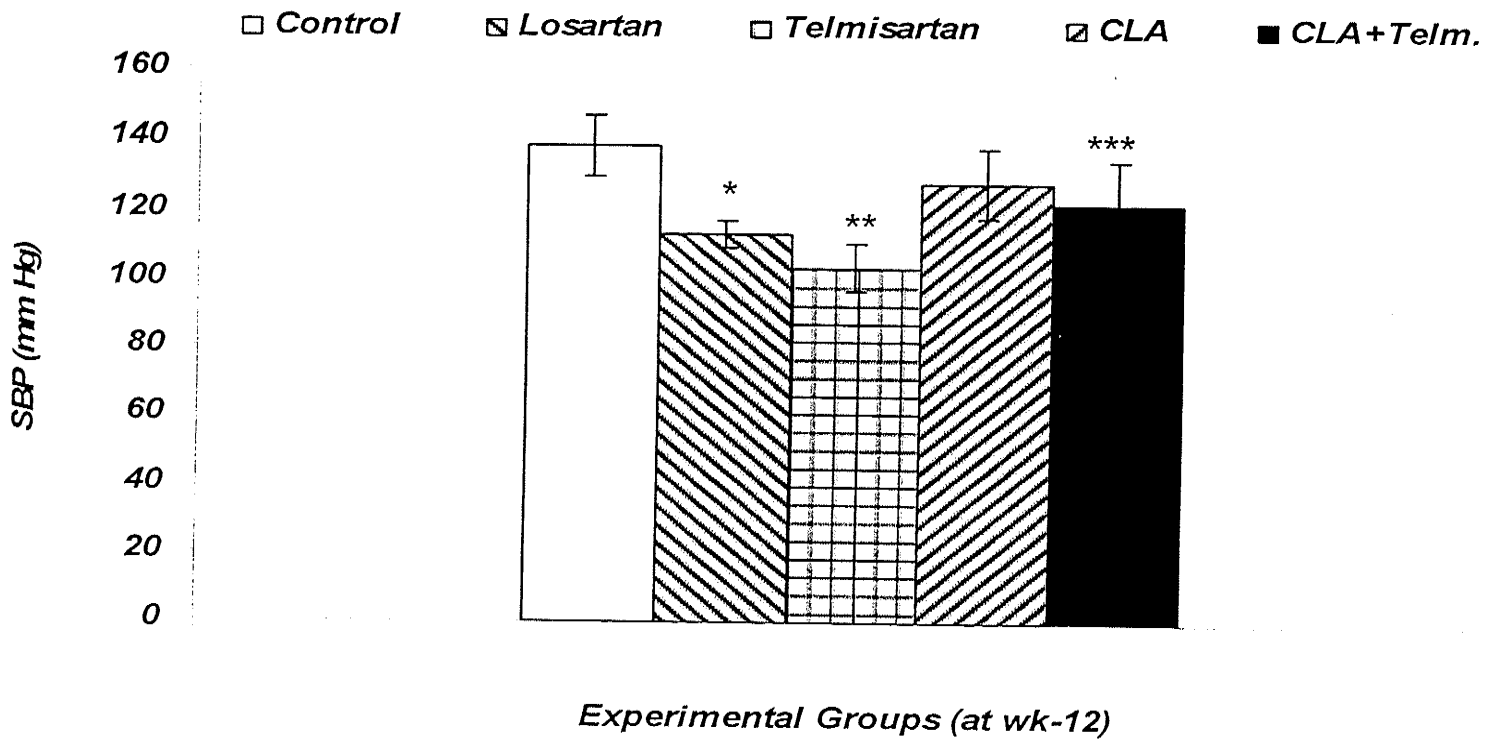


Figure 10. Effects of dietary protocols on systolic blood pressure (SBP). *Values are means ± SD, n= 7 except losartan-treated group (n= 5). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water. * indicates significant difference at p<0.001 vs. control, ** indicates significant difference at p<0.001 vs. control and CLA and p<0.01 vs. CLA+telmisartan, *** indicates significant difference at p<0.05 vs. control.*

telmisartan-treated (103.0 ± 7.0 mmHg vs. 121.0 ± 13.0 mmHg; $p<0.01$) rats. CLA-fed rats had a trend toward reducing SBP compared to the controls (128.0 ± 10.0 mmHg vs. 138.0 ± 8.0 mmHg) whereas CLA + telmisartan-treated rats exhibited a significant decrease in SBP relative to controls (121.0 ± 13.0 vs. 138.0 ± 8.0 mmHg; $p<0.05$).

6.6 ORAL GLUCOSE TOLERANCE TEST

At the end of the study, blood was drawn from tail veins and used immediately to measure glucose concentrations in the experimental groups by a glucose meter (One Touch Ultra Blood Glucose Meter; LifeScan Inc., Milpitas, CA, USA). Oral glucose tolerance testing was performed in conscious fasted-animals after oral administration of 100 mg/100 g body weight glucose by gastric gavage.

CLA + telmisartan-treated rats showed a significantly lower glucose levels at the 0 time point ($p<0.05$) as compared to controls and CLA-treated rats. No statistically significant differences were observed in the combination group at 30, 60 and 120 minutes, or among other dietary protocols (**Figure 11**).

6.7 PLASMA INSULIN LEVELS

Plasma insulin concentrations are displayed in **Figure 12**. Compared to controls (2.3 ± 0.4 ng/ml), rats of each of the CLA and telmisartan groups showed equally low plasma insulin levels (1.6 ± 0.3 ng/ml and 1.6 ± 0.7,

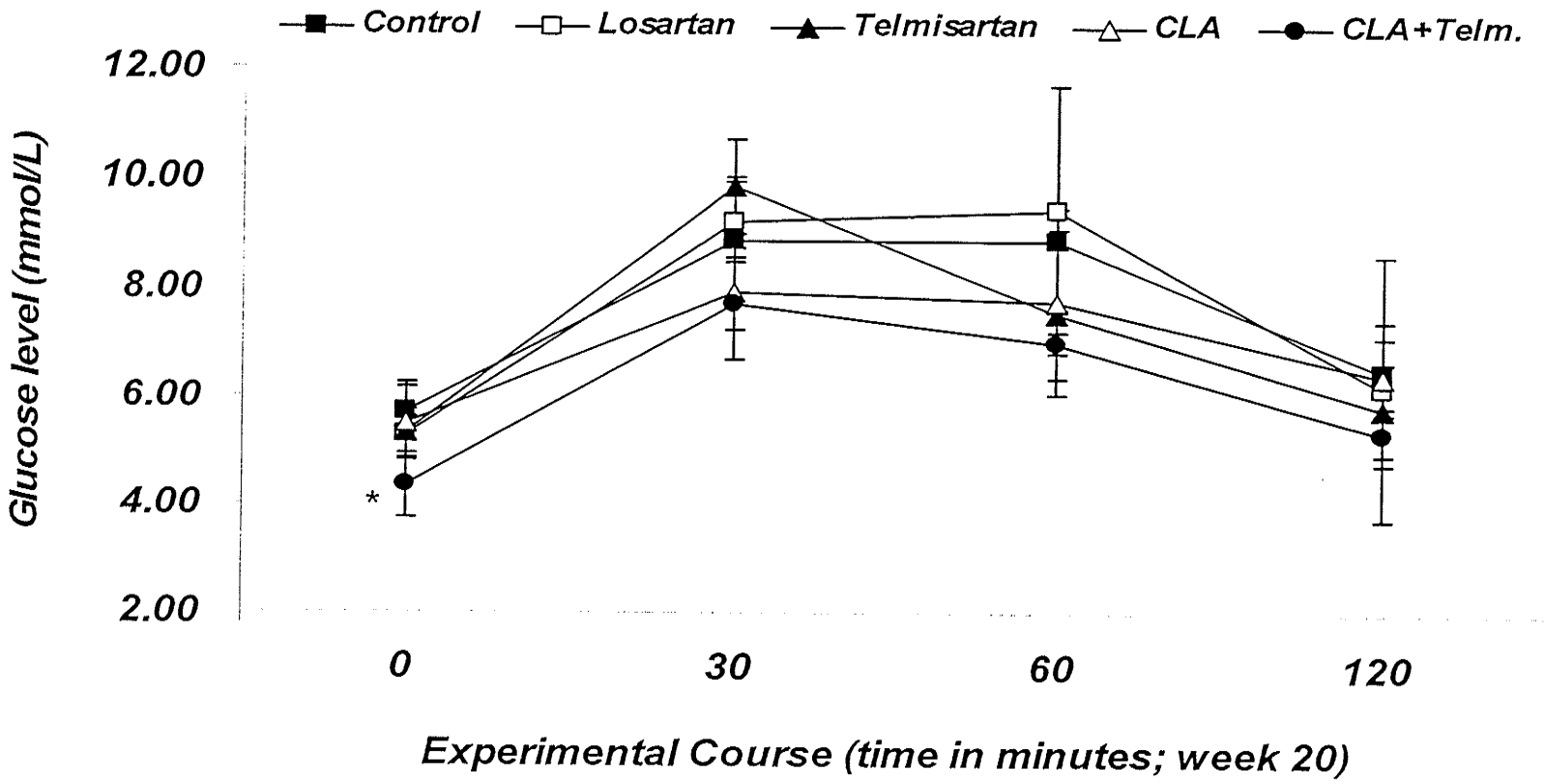


Figure 11. Effects of dietary protocols on whole-blood glucose levels of Oral Glucose Tolerance Test (OGTT). *Values are means ± SD, n= 7 except losartan-treated group (n= 5). Oral administration of glucose; 100 mg/100 g body weight by gastric gavage. CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water. * indicates significant difference at p<0.05 vs. control and CLA.*

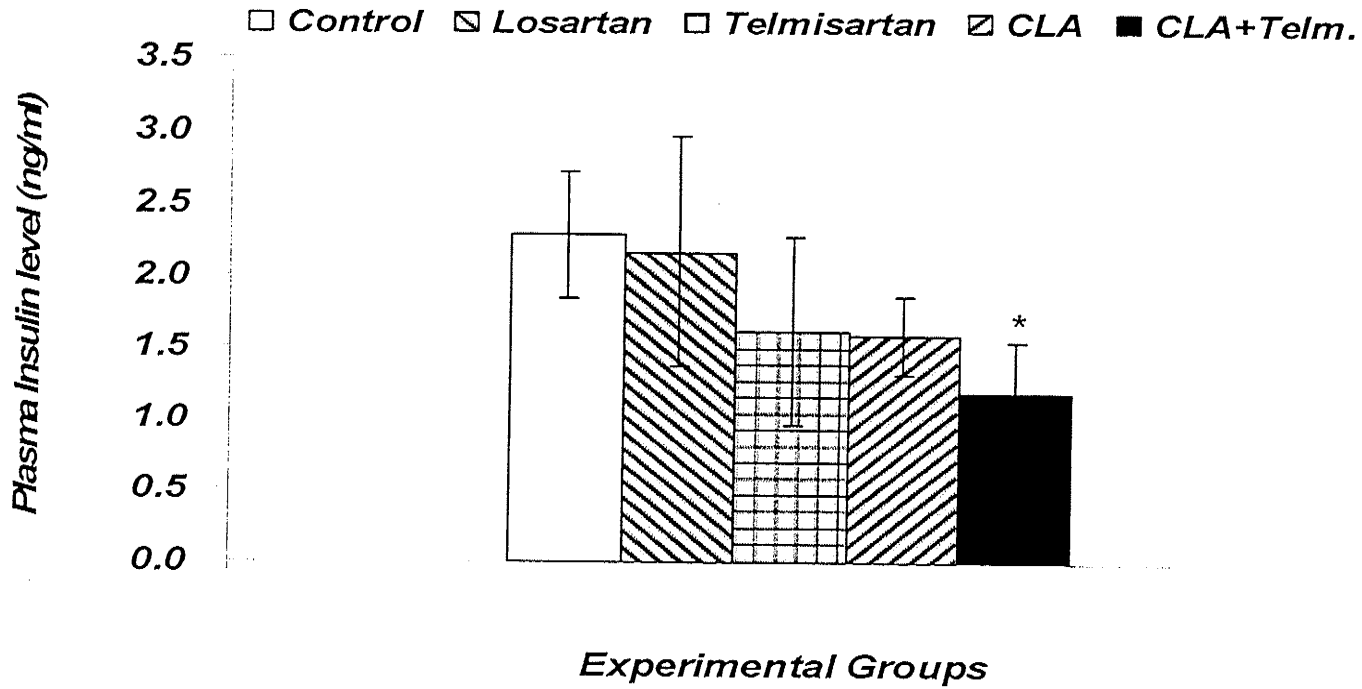


Figure 12. Effects of dietary protocols on plasma insulin concentrations. *Values are means ± SD, n= 7 except losartan-treated group (n= 5). CLA; 1.0% (w/w) diet/day of isomers c9,t11 and t10,c12 in a 50/50 ratio. Losartan and telmisartan; 5 mg/kg body wt/d in drink water. * indicates significant difference at p<0.01 vs. control and p<0.05 vs. losartan.*

respectively) by wk 20. This reduction in plasma insulin equaled 30% but was not statistically significant. CLA + telmisartan-treated rats, on the other hand, had significantly lower (1.2 ± 0.4 ng/ml; ~48%) mean plasma insulin concentrations when compared to controls ($p<0.01$) and losartan-treated ($p<0.05$) rats. The observations are consistent with previous studies on telmisartan (Benson et al. 2004) and CLA (Choi et al. 2004) in SD rats.

6.8 PLASMA CYTOKINES PROFILE LEVELS

In order to stimulate the body to produce inflammatory cytokines with a pivotal role in MetS, animals were injected ip with 400 mg LPS 2 hours before sacrifice. Plasma cytokine profiles were analyzed in pooled samples and expressed in optical density data by RayBio™ Rat Cytokine Array at the end of the study as previously described (Watanabe et al. 2005).

The alignment of cytokine arrays is shown in **Figure 13-A**. Immunoreactive staining against respective antibodies in the 5 different dietary protocols is represented in **Figure 13-B**. The positively stained 19 individual cytokine spots are clearly identifiable. While the positive control spots (a 1-2; b 1-2 and h 7-8) exhibited the highest staining intensity, the negative control (c 1-2, d 1-2) and all blank spots did not show staining above-background (**Figure 13-B**). Because samples were pooled, we were unable to perform statistical analyses for cytokine data. Regardless, we managed to calculate percentages of differences among treated groups and the control animals; a number of observations were obtained (**Table 5**). Notably, pro-inflammatory

	a	b	c	d	e	f	g	h	
1	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fractalkine	1
2	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fractalkine	2
3	GM-CSF	IFN-γ	IL-1α	IL-1β	IL-4	IL-6	IL-10	LIX	3
4	GM-CSF	IFN-γ	IL-1α	IL-1β	IL-4	IL-6	IL-10	LIX	4
5	Leptin	MCP-1	MIP-3α	β-NGF	TIMP-1	TNF-α	VEGF	Blank	5
6	Leptin	MCP-1	MIP-3α	β-NGF	TIMP-1	TNF-α	VEGF	Blank	6
7	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Pos	7
8	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Pos	8

Figure 13-A. The alignment of 19 cytokines on the Rat Cytokine Antibody Array. *Abbreviations: CINC: cytokine-induced neutrophil chemoattractants; CNTF: ciliary neurotrophic factor; GM-CSF: granulocyte macrophage-colony stimulating factor; IFN: interferon; IL: interleukin; LIX: lipopolysaccharide-induced CXC chemokine; MCP: chemokine monocyte chemoattractant protein; MIP: macrophage inflammatory protein; Neg: negative control; NGF: nerve growth factor; Pos: positive control; TIMP: tissue inhibitors of metalloproteinase; TNF: tumor necrosis factor; VEGF: vascular endothelial growth factor.*

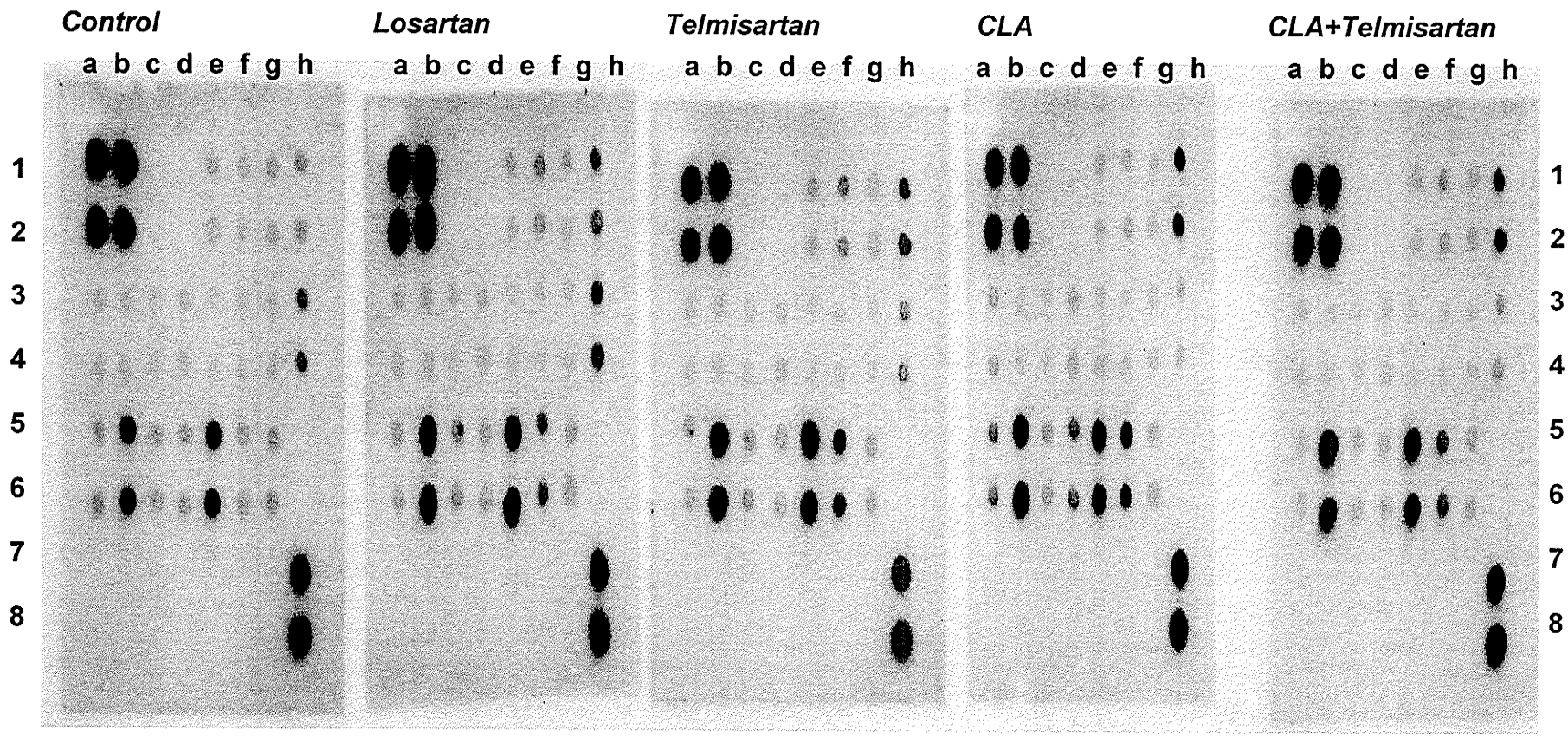


Figure 13-B. Examples of Rat Cytokine Antibody Array blots probed with the isolated protein samples. *Identification and alignment of the cytokines are illustrated in* ***Figure 13-A.*** *The protein samples were isolated at 2 h after LPS injection (400 µg/rat). Each dot represents immunoreactive staining against respective antibodies. Note the absence of staining at the negative control (1 c-d and 2 c-d) and blank slots (5 h, 6 h, 7 a-g, and 8 a-g). The relative expression levels of each cytokine were determined by comparing the staining intensity of the respective slots to that of the positive control (1 a-b, 2 a-b, 7 h, and 8 h) on the same array. Data are expressed as optical density.*

*Table 5. Effects of dietary protocols on plasma cytokine profiles at wk 20; values are optical density with background subtraction. **

Groups	Control	Losartan	% Δ to Control	Telmisartan	% Δ to Control	CLA	% Δ to Control	CLA + Telmisartan	% Δ to Control
IL1-α	659	646	-2.0%	844	+28%	821	+25%	548	-20%
IL1-β	830	939	+13%	1124	+35%	1435	+73%	1059	+28%
IL-4	469	488	+4%	536	+14%	879	+87%	726	+55%
IL-6	529	583	+10%	794	+50%	537	+15%	722	+36%
IL-10	537	676	+26%	750	+40%	1040	+94%	938	+75%
IFN-γ	976	1294	+33%	1208	+24%	987	+1%	917	-6%
TNF-α	1628	3385	+108%	5515	+239%	5680	+249%	4694	+188%
Leptin	1773	1588	-12%	1975	+11%	3686	+108%	1626	-9%

** Animals were injected with 400 μg LPS ip 2 hours before sacrifice and plasma cytokines were analyzed in pooled samples. IL1-α, interleukin 1-α; IL1-β, interleukin 1-β; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; -, less; +, more; Δ, change.*

cytokines IL1-α, IL1-β, and IL-6, as well as the anti-inflammatory IFN-γ, showed different values among experimental groups compared to the controls. IL1-α levels were slightly higher in the telmisartan- and the CLA-treated groups with 28% and 25% more, respectively, but lower in CLA + telmisartan-treated group by 20%. IL1-β levels were 35%, 73%, and 28% higher in the telmisartan-, CLA-, and CLA + telmisartan-treated rats, respectively. IL-6 levels were 50% more in telmisartan- and 36% more in CLA + telmisartan-treated groups. IFN-γ levels were only higher in losartan- and telmisartan-treated groups with 33% and 24% more, respectively.

In contrast, the two anti-atherogenic cytokines, IL-4 and IL-10, were notably increased in CLA- (87% of IL-4 and 94% of IL-10) and CLA + telmisartan-treated animals (55% more in IL-4 and 75% more in IL-10) relative to the controls.

Of interest was the apparent increase of 108% leptin level in CLA-treated, but not other groups of, animals compared to the control group. More importantly, levels of pro-inflammatory cytokine TNF-α were remarkably higher in all treatment groups relative to the controls (108% in losartan, 239% in telmisartan, 249% in CLA, and 188% in CLA/telmisartan). **Figure 14** shows summary of the quantification of relative cytokine protein levels.

6.9 VISCERAL FAT SIZE

Remarkably, animals on the CLA + telmisartan dietary protocol exhibited diminution in the visceral fat pad size relative to other groups. **Figure**

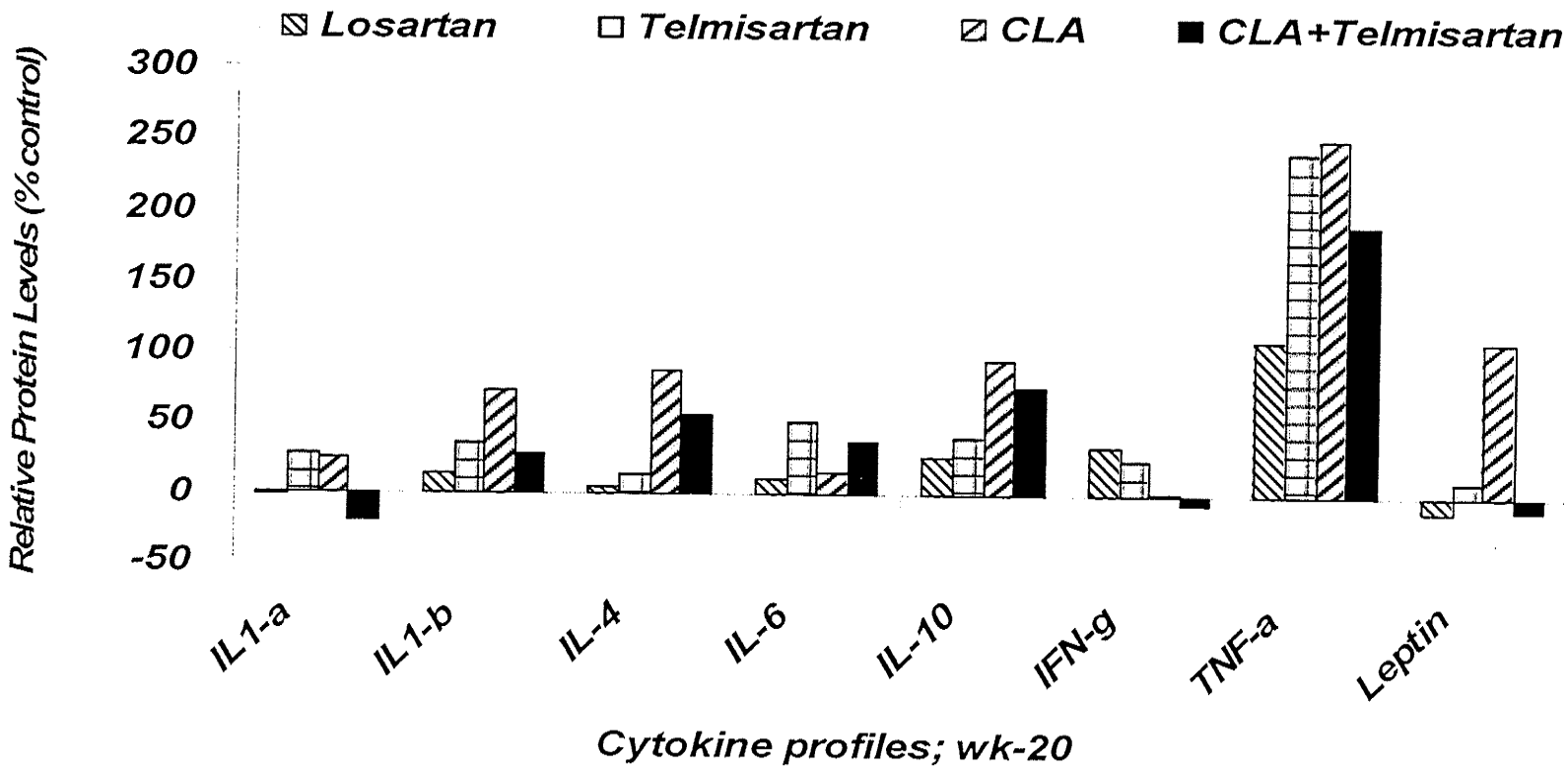


Figure 14. Summary of the quantification of relative cytokine protein levels; zero line represents control. *Values of treatment protocols are summarized in* ***Table 5.*** *Animals were injected with 400 µg LPS ip 2 hours before sacrifice and plasma cytokines were analyzed in pooled samples; no statistical interpretation could be applied. IL1-a, interleukin 1 alpha; IL1-b, interleukin 1 beta; IL-4, -6, -10, interleukin-4, -6, -10; IFN-g, interferon gamma; TNF-a, tumor necrosis factor alpha.*

15 shows representative photographs of visceral fat at wk 20. The notable effect of the CLA/telmisartan co-therapy on the adipose tissue might be a reason for the other actions of the combination treatment.

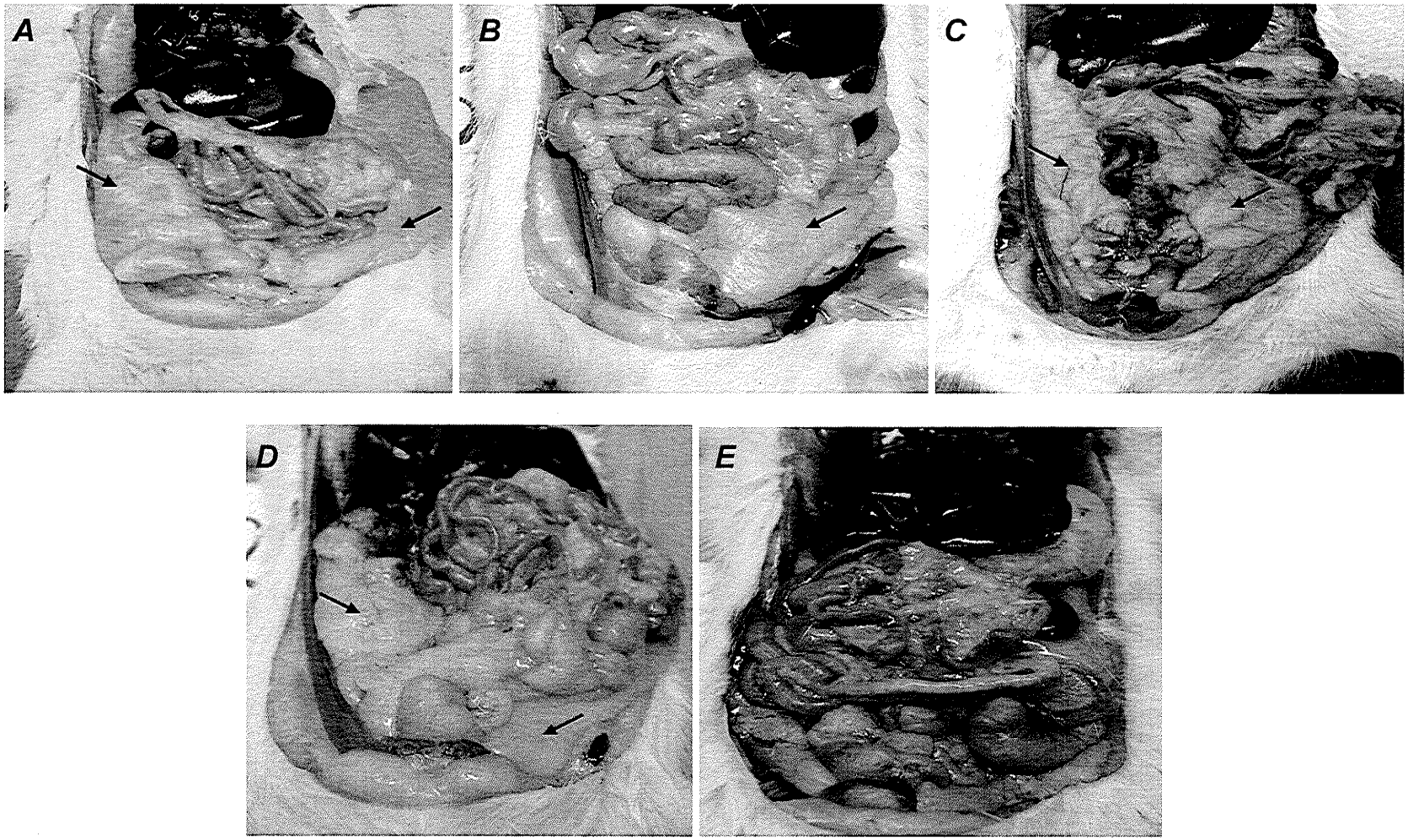


Figure 15. Effects of dietary protocols on visceral fat pad size; representative photographs of visceral fat at wk 20. ***A****, control;* ***B****, losartan;* ***C****, telmisartan;* ***D****, CLA;* ***E****, CLA + telmisartan. Arrows indicate abdominal adipose tissue.*

7.0 DISCUSSION

7.1 SUMMARY OF CURRENT STAGE OF KNOWLEDGE AND HYPOTHESIS

Obesity, IR, hyperglycemia, elevated BP, and atherogenic dyslipidaemia characterized by hypertriglyceridemia, low concentrations of HDL-cholesterol and small, dense LDL particles are among the major risk factors for CHD. Patients with MetS (abdominal obesity, dyslipidemia, hyperinsulinemia, hypertension, and a state of proinflammation) are at higher risk for CHD and diabetes (Laaksonen et al. 2002), and have increased CVD-related mortality (Isomaa et al. 2001; Lakka et al. 2002). This higher risk is further amplified if it is accompanied by a constellation of other risk factors, namely sedentary lifestyle, unhealthy diet, family history, male gender, postmenopausal status, aging, and smoking. Improvements in lipid, carbohydrate, and energy metabolism by lifestyle modification, dietary and/or pharmacological agents have been shown to reduce morbidity and mortality from cardiovascular events.

To date, there are two potential therapeutic approaches for MetS. One strategy is to identify and treat each risk factor separately, unrelated to its clustering with other risk factors. This strategy will continue to be part of effective therapies, and exclusive targeting of single risk factors with drugs is advocated as the only realistic approach by some authorities (Kahn et al. 2005). Another strategy is available to target multiple risk factors simultaneously (Grundy et al. 2005).

Examples of candidate drugs-of-choice in treating MetS based on the first approach (that is, to treat each risk factor separately) are orlistat and sibutramine (indicated for obesity), fibrates or statins for hyperlipidemia, ACEI and ARB families to treat hypertension, and metformin and the family of TZDs for diabetes. Although these drugs seem effective in preventing morbidity and mortality from cardiovascular events, their adverse effects and the associated polypharmacy problems may be a limiting factor in patients with the MetS. For example, drug-induced myopathy and rhabdomyolysis as well as hepatic toxicity are among serious adverse effects for fibrates and statins whereas weight gain is a major side effect of TZD treatment.

At present, there are no approved drugs that can reliably reduce all of the MetS risk factors over the long term, and so there is growing interest in therapeutic strategies that might target multiple risk factors more effectively. One drug approved for treatment of essential hypertension, telmisartan (Micardis®), is a novel alternative candidate (that is, to target all or multiple risk factors with a single therapy) due to its anti-hypertensive and anti-glycaemic actions. Telmisartan manifests selective PPARγ-modulating activity with 25-30% of the maximum level achieved by the conventional full agonists, such as pioglitazone and rosiglitazone, in transactivation assays (Benson et al 2004). Administration of telmisartan has resulted in a significant attenuation of weight gain, and serum glucose, insulin, and TG levels (Benson et al. 2004) in rats fed high-carbohydrate, high-fat diet. Moreover, telmisartan caused a dose-dependent increase in adipocyte lipid-binding protein (aP2) and adiponectin

mRNA levels, both PPARγ-dependent target genes, and augmented GLUT4 protein expression, and basal and insulin-stimulated 2-deoxy glucose uptake in differentiated 3T3-L1 adipocytes (Fujimoto et al. 2004). This dual-action ARB/PPARγ drug is reminiscent of PPARγ/PPARα drugs, and could be a prototype for future agents to treat the MetS (Kurtz 2005).

On the other hand, global interest in alternative medicine, nutraceuticals, and functional foods has grown recently. CLA is a naturally occurring omega-6 FA that is primarily found in ruminant meat and dairy products. Similar to telmisartan, several investigations have shown that CLA shares functional similarities with ligands of PPARs, most notably the PPARα (Moya-Camarena et al. 1999a; Moya-Camarena et al. 1999b) and PPARγ (Houseknecht et al. 1998; Yu et al. 2002) subtypes. CLA is reported in the literature to reduce body weight, hypertension, and diabetes, and to improve feed efficiency, energy metabolism and immune functions. Therefore, it was reasonable to hypothesize that the addition of CLA to telmisartan therapy will be an effective way to achieve greater amelioration in individual components of MetS as compared to controls, or single telmisartan or CLA therapy.

7.2 EFFECTS OF DIETARY CLA AND TELMISARTAN CO-ADMINISTRATION

The primary objective of the present study was to determine the response in the adult SD rats to dietary supplementation with CLA and telmisartan for several parameters of MetS. Herein, we provide the first

evidence that a co-administration of dietary CLA and the ARB/PPARγ partial agonist telmisartan may act as a promising novel therapy for MetS; attenuating high body weight, reducing hyperlipidemia and hypertension, improving glucose and normalizing insulin, and, to a lesser extent, glucose tolerance metabolism, thus preventing or delaying the onset of MetS in the SD rat model.

7.2.1 Body Weight

The initial concept of MetS (Reaven 1988) did not include obesity as one of its components. Abdominal obesity is, nevertheless, one of the key features that accompany IR and its dysmetabolic state (Després 1993; Després 1998; Lamarche et al. 1998). Obesity affects the metabolism of lipids and glucose, the regulation of BP, thrombotic and fibrinolytic processes, and inflammatory reactions (Grundy 2004). It must, however, be emphasized that obesity alone is probably inadequate for onset of full-blown MetS (Vega et al. 1996). Despite this fact, weight reduction may reduce all of the metabolic risk factors in abdominally obese individuals with MetS (Grundy 2006).

Some studies in rodent models have consistently shown reductions of weight gain by feeding CLA (West et al. 1998; Park et al. 1999a; Ryder et al. 2001; Terpstra et al. 2002). Others have not shown any effect (Park et al. 1997; West et al. 2000; Sisk et al. 2001; Pariza et al. 1999), most likely due to either low levels (≤0.5% in the diet) of CLA (West et al. 1998; Sisk et al. 2001) or low concentrations of the t10,c12 isomer in CLA mixtures (Park et al.

1999b; Ryder et al. 2001). Results of studies in animals other than rodents are controversial. For example, reports in pigs have shown increase (Thiel-Cooper et al. 2001; Bee 2000), decrease (O'Quinn et al. 2000), or no effect (Ostrowska et al. 1999; Tischendorf et al. 2002; Wiegand et al. 2002) of CLA on body weight. In studies that have used mouse models prone to obesity, or the ZDF rat model, CLA-mix- or the t10,c12-CLA–treated animals gained less body weight than did matched controls (Tsuboyama-Kasaoka et al. 2000; Ryder et al. 2001; Park et al. 1997; Houseknecht et al. 1998; West et al. 1998, DeLany et al. 1999). In humans, most of the studies have not reflected the dramatic findings obtained in animal studies. One of the first studies in healthy adult women examined the effects of 3 g/day intake of CLA for 64 days, and reported no differences in body composition or percentage fat mass compared to SFO placebo (Zambell et al. 2000). Similarly, Kreider et al. (2002) reported no effects of CLA (6 g/day, 28 days) on body composition or body fat mass in healthy excerising humans. However, two studies from Norway in healthy exercising humans (CLA, 1.8 g/day) and in overweight and obese humans (CLA, 1.7, 3.4, 5.1, and 6.8 g/day) for 12 weeks showed that CLA can decrease fat mass without significantly affecting body weight (Thom et al. 2001; Blankson et al. 2000).

In the model we used, CLA-mix treated animals produced no reduction in weight gain. This finding is consist with a previous study by Yamasaki et al. (1999), in which administration of 1.0% mixed CLA isomers (34.7% c9,t11 and 35.6% t10,c12) with normal diet over 3 weeks in SD rats did not decrease the

body weight gain. Similarly, Azain et al. (2000) reported no effects on body weight by feeding 0.25-0.5% of CLA mix-isomers for 5 weeks. While there were no differences in food intake, a reduction in fat mass weights was observed in CLA-treated SD rats, and referred to as reduction in cell size rather than a change in cell number (Azain et al. 2000). In another study with the same rat model and regular diet, Yamasaki et al. (2000) demonstrated a lack of effect of 2.0% CLA-mix on body weight in 12 weeks. The effects of CLA on body composition in the SD rat model seem to be isomer-dependent since in the study by Choi et al. (2004), which lasted for 8 weeks, body weight gain was significantly decreased by c9,t11-CLA, but not t10,c12 or CLA-mix. Choi's study, accordingly, concluded that the significant decrease in body weight gain might be attributed to a specific effect of CLA on energy expenditure rather than to a change in energy intake since no significant differences in food intake could be observed.

In our study, and in contrast to the CLA-treated group of rats, telmisartan-treated rats showed a trend toward reducing body weight; by ~10% in wk 5 and 16% by wk 20 as compared to controls. These findings assent with data reported by Benson et al. (2004) in which oral administration of telmisartan significantly attenuated the weight gain (~10% less) in 5 weeks. There, researchers (Benson et al. 2004) considered telmisartan's partial PPARγ-modulating activity as one possible mechanism of action for their observations.

In our 20-week long study, we observed an additive reduction in body weight when CLA was co-administered with telmisartan. More importantly, this reduction in weight gain was not associated with significant reduction in food intake among the dietary protocols. Basically, therefore, the exciting 31% attenuation of weight gain induced by the CLA/telmisartan protocol can be attributed to effects on energy expenditure rather than reduction in appetite and energy intake. Of course, because of the difficulties in measuring food intake with 100% accuracy, the possibility that group differences in energy intake might be contributing to the group differences in body weight cannot be completely ruled out.

Effects on uncoupling proteins (UCPs) which are related to, and involved in, regulation of energy expenditure and synthesis might be a possibility. UCP-1, -2, and -3 play an important role in mitochondrial electron transport, diet-induced thermogenesis, control of production of reactive oxidative stress, and regulation of FA oxidation. Thus, the upregulation of UCPs in tissues plays a crucial role in increasing energy expenditure and, may also affect insulin sensitivity. The decreased weight gain in c9,t11-CLA fed SD rats observed by Choi et al. (2004) was accompanied by an increase in UCP-2 mRNA levels. Similar observations have been reported in a study by Ryder et al. (2001), in which CLA intake increased gene expression of UCP-2 and UCP-3 in ZDF rats. To date, no studies have examined the effects of telmisartan on UCPs; future research could provide conclusive data.

Activation of transcription factors that are involved in insulin sensitivity, PPARα, PPARγ, and SREBP-1c (Shulman 2000; Stears et al. 2001; Yoon et al. 2001), might represent another possible mechanism for our observations. PPARs can modulate lipid homeostasis, including absorption, synthesis, storage, transport, and oxidation of lipid substrates, by regulating the transcription of LPL, carnitin palmitoyltransferasle (CPT), fatty acid binding protein (FABP), peroxisomal and mitochondrial β-oxidizing enzymes, and apolipoproteins (Lee et al. 2003). Activation of PPARα has been shown to alter transcription of many of these proteins, resulting in a significantly increased release of FAs from adipose tissue, FA transport, and oxidation of FAs, and a subsequent decrease in serum lipid levels (Takahashi et al. 2001). With reports suggesting that CLA may decrease body fat and serum lipids by activating PPARα–dependent gene expression, and others suggesting weak activity of telmisartan on PPARα, we wonder whether CLA/telmisartan co-administration increases activation of PPARα. CLA, at least, has previously shown high affinities for PPARα activation and induced accumulation of PPAR-responsive mRNAs in a rat hepatoma cell line (Moya-Camarena et al. 1999a). Previously, telmisartan exhibited weak activity on PPARα (Benson et al. 2004).

PPARγ plays a major role in the storage of FFAs in adipose tissues by stimulating the lipolysis of circulating TGs and subsequent uptake of FFAs into adipocytes. Decreasing circulating FFA levels improves glucose metabolism, inhibits gluconeogenesis, and decreases IR (Jiang et al. 2002). Choi's study

(2004) showed a trend of CLA diets toward increasing PPARγ, but not PPARα, mRNA levels in adipose tissue and, accordingly, suggested that CLA does not affect the regulation of PPARγ and PPARα, at least in regard to the amount of mRNA transcript.

On the other hand, Benson et al. (2004) have demonstrated ability of telmisartan to function as a partial agonist (mixed agonist/antagonist) of PPARγ; altering expression of the target genes and reducing glucose and TG levels in SD rats. Moreover, telmisartan concentrations of 25 μmol/L, but not <10 μmol/L, caused a 4-fold modest activation of PPARα expression in cell-based transactivation assays (Benson et al. 2004). Thus, it seems possible that telmisartan may also activate PPARα and increase expression of its target genes.

7.2.2 Visceral Fat Size and Leptin Levels

In patients with MetS and/or diabetes, increased visceral fat is believed to play an important role in pathogenesis of IR and dyslipidaemia (Pi-Sunyer 2004). Evidence suggests that surgical removal of intra-abdominal visceral fat can improve insulin sensitivity (Gabriely et al. 2003). Additionally, it seems that pharmacological treatments that redistribute fat from visceral to subcutaneous depots, such as the conventional PPARγ agonists TZDs, can also improve IR and dyslipidaemia (Kawai et al. 1999; Rausouli et al. 2005). In the present study, we observed that CLA/telmisartan-treated rats had notably less visceral fat which correlated with the decreased body weight relative to all other

groups. This effect of the combination therapy appears consistent with some previous studies examining both CLA and telmisartan.

Dietary CLA decreases adiposity in different animal models including mice (Park et al. 1997; West et al. 1998; West et al. 2000; DeLany et al. 1999), rats (Ryder et al. 2001; Sisk et al. 2001; Yamasaki et al. 2003), hamsters (de Deckere et al. 1999), and pigs (Ostrowska et al. 1999; Thiel-Cooper et al. 2001). Of these, male SD rats fed diets containing 4, 7 or 10% added fat, supplementation of 1.5% CLA for 3 weeks significantly reduced white adipose tissue weight (Yamasaki et al. 2003). Similar results have been obtained in female SD rats supplemented with 0.25 or 0.5% CLA for 5 weeks (Azain et al. 2000). A reduction in fat pad mass has also been observed in ZDF rats when they were fed 1.5% CLA (Ryder et al. 2001). One study of double blind design on human subjects further supports results of studies conducted in animals. Data presented by Mougios et al. (2001) indicated that supplementation of 0.7–1.4 g of CLA daily for 4–8 weeks modulates body fat and serum lipids. Percentage body fat calculated from skinfolds and fat mass was significantly reduced in the CLA group relative to placebo. Other clinical trials, however, do not support these findings (Zambell et al. 2000; Kreider 2002; Malpuech-Brugere et al. 2004).

Mechanisms via which CLA affects body composition vary and remain elusive. In different *in vivo* and *in vitro* studies, CLA has been shown to decrease energy intake (West et al. 1998) or increase energy expenditure (West et al. 2000), reduce fat cell size (Azain et al. 2000), alter preadipocyte

differentiation (Evans et al. 2000; Brodie et al. 1999), increase apoptosis of adipocytes (Yamasaki et al. 2003; Akahoshi et al. 2002), inhibit lipogenesis in the liver and adipose tissue (Park et al. 1999b), and increase FA oxidation (Sergiel et al. 2001).

One recent study by Sugimoto et al. (2006) has provided evidence for the ability of telmisartan to reduce the accumulation of visceral fat and decrease adipocyte size in SD rats to a greater extent when compared to counterpart controls. In the same study, telmisartan increased the expression of both nuclear-encoded and mitochondrial-encoded genes known to play important roles in mitochondrial energy metabolism. The observations looked consistent with the hypothesis of Sharma et al. (2002) that angiotensin II receptor blockade per se may affect adipocyte biology and promote the formation of small, metabolically efficient adipocytes. Based on other reports in literature (Berger et al. 2003; Kubota et al. 1999), Sugimoto et al. (2006) raised the possibility that the effects of telmisartan on weight gain and adipocyte size might be also related, at least in part, to its ability to function as a partial agonist of PPARγ.

Obesity is usually accompanied by abnormalities in leptin and insulin secretion together with defects in lipid and carbohydrate metabolism (Buettner et al. 2000; Zhou et al. 1998). Serum leptin levels may correlate with body fat and body mass index (Maffei et al. 1995; Frederich et al. 1995). Some studies have shown that CLA can reduce levels of leptin in rats, mice, and humans on low fat diet (Delany et al. 1999, Medina et al. 2000; Yamasaki et al. 2000;

Masuzaki et al. 1995). In the present study, dietary CLA increased plasma leptin levels by ~108% as compared to the controls. Some studies in rodents (Zhang et al. 1996) and an adipocyte cell line (Kallen et al. 1996) have demonstrade that activation of PPARγ decreases leptin gene expression. In a study conducted in SD rats fed a 60% fat diet, hyperleptinemia was associated with an increase in mRNA of a PPARα protein and FA oxidation (Lee et al. 2001). This finding was further supported by a very recent study in which up-regulation in the expression of PPARα and some of its target genes in the liver was associated with hyperleptinemia and obesity in both rats and mice (Hsu et al. 2006). For those animals that did not develop obesity in the same study, changes in the liver PPARα signaling were only minimal. These results raised the possibility that obesity-associated hyperleptinemia may have a role in the induction of PPARα gene expression (Hsu et al. 2006).

7.2.3 Lipid Metabolism

To assess whether the CLA/telmisartan-mediated changes in body weight were associated with changes in lipid metabolism, serum lipid profiles were quantified. Isomers of CLA have been shown to alter lipid metabolism in animal, human, and cell culture studies via a number of proposed mechanisms, including decreased cholesterol absorption (Sher et al. 2003) and alterations in arachidonic acid-mediated eicosanoid metabolism (Belury 2002). It has also been suggested that CLA could reduce LDL-cholesterol by inhibiting the secretion of apolipoprotein B (Yotsumoto et al. 1999) or by

enhancing the clearance rate of circulating LDL by increasing LDL receptor activity (Grundy et al. 1990). Thomas et al. (2000) stipulated that the cholesterol-reducing effect seen in CLA-fed hamsters is mediated in part by its inhibitory effect on cholesterol absorption through the down-regulation of intestinal acyl CoA:cholesterol acyltransferase (ACAT) activity.

In the present study, only rats receiving the CLA/telmisartan combination exhibited a significant reduction in TC as compared to the controls. In general, data regarding effects of CLA on lipid metabolism in SD rats are limited. One recent study by Kloss et al. (2005) provided evidence that supplementation of 1.5% CLA-mix lowers serum TC, but not TG or HDL-cholesterol, concentrations in 4 week period. Specifically, TC was lower only in CLA-supplemented animals fed a diet rich in saturated FAs. Since HDL-cholesterol levels were not significantly different in the same study, the differences in TC levels were apparently due to the concentrations of cholesterol in lower density fractions of lipoproteins. Studies, along with Kloss et al. (2005), suggest that lipid-lowering effect of CLA seems to be partly dependent on the nature of the dietary fat and animal species (Ealey et al. 2002; Nagao et al. 2003a). Because hepatic FA synthesis is strongly down regulated by PUFAs, the basal rate of hepatic lipogenesis may be different between these experiments and this may explain the divergent results.

The effects of telmisartan on plasma TC levels have been assessed in few clinical trials, with no adverse effects (Michel et al. 2004) and significant improvement (Derosa et al. 2004a; Derosa et al. 2004b) reported.

High fructose feeding causes diet-induced alterations in lipid metabolism and decreases insulin sensitivity in SD rats, a hallmark of which is a rapid and profound hypertriglyceridemia (Kelley et al. 2005). One of the mechanisms that contributes to serum hypertriglyceridemia in this model is suppression of hepatic PPARα. HMG-CoA inhibitors, which reduce serum TGs in these animals, also elevate/restore hepatic PPARα (Kelley et al. 2005). Hepatic fructose metabolism leads to precursors of TG synthesis, such as leading to generation of TGs.

In the present study, in agreement with previous reports (Benson et al. 2004; Catena et al. 2003; Ackerman et al. 2005), we observed a dramatic rise in serum TG levels owing to the fructose-rich diet. Both telmisartan administration and, to a greater extent, CLA + telmisartan co-administration significantly reduced serum TG levels in our study. These findings are consistent with previous observations with telmisartan-treated spontaneously hypertensive (Li et al. 2006) and telmisartan-treated SD (Benson et al. 2004) rat models, and CLA-treated SD rats (Yamasaki et al. 1999) suggesting an additive effect of the combination therapy.

We also observed a lack of HDL-raising effects of CLA/telmisartan co-administration. Also, telmisartan- and CLA + telmisartan-treated rats did not show significant reduction in the LDL-cholesterol levels. Whereas no studies have investigated effects of telmisartan, alone, on serum LDL-cholesterol in SD rats, few clinical trials (Derosa et al. 2004a; Derosa et al. 2004b) have demonstrated a significant improvement.

It is known that CLA activates PPARα, and that hepatic PPARα activation is involved in regulation of energy balance, lipid homeostasis and body weight gain (Fruchart 2001; Fruchart et al. 2001). Therefore, the mechanism underlying body weight and lipid lowering effects of CLA/telmisartan co-therapy might be related to PPARα activation. This concept is further supported by Costet's study (Constet et al. 1998) in which deletion of PPARα gene in mice caused abnormalities in TG and cholesterol metabolism and, eventually led to obesity.

7.2.4 Glucose and Insulin Metabolism

Disorders of glucose metabolism are intimately related to the MetS. The relation between impaired fasting glucose or impaired glucose tolerance, and IR is well supported by clinical trials and rodent studies (Eckel et al. 2005).

To find out whether consumption of CLA and telmisartan could alter glucose homeostasis and insulin action, we determined the effects of dietary treatment on circulating blood glucose and insulin concentrations as well as glucose tolerance. Results obtained in various rodent models concerning the effects of CLA on insulin sensitivity are controversial. Data in the literature show that CLA improves insulin sensitivity in Zucker *fa/fa* rats, an animal model that shows either severe IR or T2DM (Henriksen et al. 2003; Houseknecht et al. 1998; Ryder et al. 2001; Nagao et al. 2003b), and in diabetic mice (Hamura et al. 2001). In contrast, CLA induces IR in C57BL/6J mice, an animal model not showing previous glucose metabolic disturbances

(Tsuboyama-Kasaoka et al. 2000; Clément et al. 2002; Roche et al. 2002). These results suggest that the effects of CLA on insulin sensitivity depend on species and/or metabolic status.

Houseknecht et al. (1998) was first to report ability of CLA to normalize impaired glucose tolerance and improve hyperinsulinemia in ZDF rats due, at least in part, to the activation of PPARγ. The effects of CLA on insulin sensitivity, in addition to its agonist actions at PPARγ, could be attributed to multiple mechanisms. For example, CLA's anti-diabetic effect may be due to its effect on lipid metabolism, including FA oxidation, lipolysis, de novo lipogenesis, and the expression of enzymes involved in lipid metabolism (Evans et al. 2002).

Another observation from the present study is that a "high-fructose diet" does not significantly increase serum glucose in SD rats. Despite this observation, losartan-, telmisartan-, and CLA + telmisartan-treated rats showed a hypoglycemic effect. In agreement with our observations, previous studies did not show a better effect of losartan over telmisartan on serum glucose in SD rats (Benson et al. 2004) or human subjects (Vitale et al. 2005). Losartan, however, has been shown to ameliorate obesity-induced hyperglycemia in *db/db* mice in a dose-dependent manner (Chu et al. 2006) and, further, showed inhibitory properties on glucose-induced effects in an *in vitro* study (Li et al. 2006).

Basically, the observed 30% reduction in serum glucose concentrations by the CLA/telmisartan combination might be attributed to actions of both

telmisartan and CLA. Benson et al. (2004) reported a 12% reduction in glucose levels by telmisartan administration. As well, Choi et al. (2004) showed that c9,t11 and t10,c12 CLAs markedly decreased serum glucose, enhanced glucose tolerance, and decreased blood insulin. Choi et al. (2004) demonstrated that rats fed CLA had enhanced insulin sensitivity and, overall, all the CLA treatments decreased IR as estimated by the IR index. Whether the effects of CLA/telmisartan combination are additive or completely unrelated to their individual effects needs to be determined, and could represent potentially fruitful areas of future research.

Increased tissue and plasma TG and FFA contents have been reported to interfere with insulin-stimulated signaling pathways and the subsequent translocation of GLUT4 and glucose uptake, which leads to IR and gluconeogenesis (Shulman 2000). In the present study, CLA/telmisartan treatment reduced serum concentrations of TG. This, in turn, probably reflected the glucose lowering effect of the combination treatment.

Plasma insulin concentrations were high in the high-fructose (control) group, low in CLA- and telmisartan-treated rats, and even lower in the CLA/telmisartan combination group. This is consistent with previous studies on telmisartan (Benson et al. 2004) and CLA (Choi et al. 2004) in SD rats, suggesting possible involvement of PPARs activation.

Nagai et al. (2002) demonstrated that a diet high in fructose suppresses PPARα expression in the liver. Because insulin is reported to negatively regulate PPARα expression (Steineger et al. 1994), it is possible that the

higher insulin levels we observed in the control group partly contributed to the decreased expression of PPARα. More importantly, our observation that CLA + telmisartan caused a significant reduction in insulin levels further supports the possibility that CLA/telmisartan combination activated the PPARα expression. Interestingly, Nagai et al. (2002) reported that mRNA levels of PEPCK, a rate-limiting enzyme of gluconeogenesis, was decreased in their fructose-fed rats, and that fenofibrate treatment did not change the expression of PEPCK in control rats. Accordingly, they suggested that the improvement in insulin sensitivity, they observed, with fenofibrate (a PPARα agonist) treatment may be independent of the regulation of hepatic glucose production. Nagai et al. (2002) observed no significant elevation in glucose levels with high-fructose diet and observed no significant reduction by the fenofibrate. Altogether, it seems that activation of PPARα improves the insulin sensitivity but does not significantly affect glucose metabolism in SD rats. Given the fact that CLA/telmisartan co-therapy improved both insulin and glucose metabolism, it seems reasonable to suggest that the effects of the combination were beyond the activation of PPARs alone.

7.2.5 Systolic Blood Pressure

Clinical studies show that high BP is prevalent in obesity and in diabetes; both conditions are usually associated with IR (Ferrannini et al. 1987). To test this and assess if consumption of CLA and telmisartan prevents hypertension, we determined the effects of dietary treatment on BP. To date,

CLA has shown anti-hypertensive effects in studies conducted in Otsuka Long–Evans Tokushima fatty (Nagao et al. 2003a), Zucker diabetic fatty (Nagao et al. 2003b), and spontaneously hypertensive (Inoue et al. 2004) rats. Of these, Nagao et al. (2003a,b,c) provided evidence that a CLA-mix (50:50) or the t10,c12, but not c9,t11 isomer decreases BP and hypertension in various rat models prone to develop obesity, diabetes and obesity together, or hypertension. The effect was attributed to regulation of adipocytokine production in obese animals.

To the best of our knowledge, this study is the first to explore effects of CLA on BP in the SD rat model. Herein, we sought to specifically measure SBP owing to the fact that elevation of SBP predicts the risk of CVD better than increases in DBP (Kannel et al. 1969). As well, SBP has been reported to be easier to determine, it allows more appropriate risk stratification than DBP, and finally, the benefits of treating SBP have been well documented by Systolic Hypertension in the Elderly Program (SHEP; 1991) and others (Staessen et al. 1997).

As expected and in agreement with data reported by Sharpe et al. (2001) and Brenner et al. (2001), the two ARB drugs resulted in a significant reduction in SBP in treated rats when compared to the controls. Administration of CLA alone did not result in a significant reduction in SBP whereas the CLA/telmisartan co-administration significantly reduced SBP relative to the controls. The addition of CLA to telmisartan, however, masked the anti-hypertensive effects of the ARB and diminished the SBP lowering effects of

telmisartan when compared to the effect of telmisartan alone. Further investigations are needed to identify the mechanisms of these findings.

One possible mechanism of action may be related to the effect of CLA on adiponectin. Several reports suggest that adiponectin has anti-diabetic and anti-atherogenic properties, and serves as an important modulator for metabolic and vascular diseases (Ouchi et al. 2000; Ouchi et al. 2001). Recently, it has been reported that plasma adiponectin concentration in patients with essential hypertension was significantly lower than in normotensive healthy subjects (Kazumi et al. 2002; Adamczak et al. 2003; Iwashima et al. 2004). Moreover, adiponectin-knockout mice have been reported to develop hypertension (Ouchi et al. 2003). These results suggest that plasma adiponectin is an independent regulatory factor for hypertension.

Previous reports indicated that TZDs, known to be ligands for PPARγ, increase the levels of adiponectin mRNA through the activation of its promoter (Maeda et al. 2001). It is well documented that CLA activates PPARγ in adipose tissue from rats (Houseknecht et al. 1998). Therefore, since CLA/telmisartan did not show additive effect on BP, it is possible to conclude that the combination therapy neither resulted in more PPARγ activation nor enhanced adiponectin expression.

7.2.6 Plasma Cytokines

Several recent studies suggest that MetS is also associated with an increase in inflammatory cytokines (Ridker et al. 2004). For that reason, we

sought to explore the relation between CLA and telmisartan consumption and inflammatory markers; plasma cytokine profiles were determined at the end of the study and percentages of differences of protein levels between multiple groups and the control were obtained.

Cells of the immune system secrete cytokines during the nonspecific immune response to give directions and contributions to inflammatory reactions. In this respect, the pro-inflammatory cytokine TNF-α is a key mediator. Our data do not fully support previous reports in which CLA decreased production of TNF-α in human and animal models (O'Shea et al. 2004). In our study, levels of the TNF-α were remarkably increased in all treatment groups, including CLA, as compared to the controls.

Anti-atherogenic cytokines, IL-4 and IL-10, but not INF-γ, were notably increased in CLA- and CLA + telmisartan-treated animals relative to the controls. These observations are consistent with previous studies in CLA suggesting that CLA enhances immune functions (Turek et al. 1998; Hayek et al. 1999; Wong et al. 1997; Yang et al. 2000; Bassaganya-Riera et al. 2001).

7.3 A SUMMARY OF MAJOR FINDINGS OF THE STUDY AND CONCLUSIONS

7.3.1 Study Major Findings

1. Combination of CLA-mix isomers with telmisartan of 5 mg/kg body wt/d is safe and well-tolerated in SD rats over 20 weeks.

2. Co-therapy of CLA and telmisartan significantly attenuated high body weight in 20 weeks when compared to control, losartan-, and CLA-treated, but not the telmisartan-treated, rats.
3. The attenuation in body weight by the CLA/telmisartan co-administration was not accompanied with significant differences in food and water intakes.
4. CLA/telmisartan dietary protocol significantly reduced serum levels of total cholesterol over 8 weeks relative to controls and losartan-treated, but not the CLA- and the telmisartan-treated, rats.
5. CLA/telmisartan dietary protocol significantly reduced serum levels of triglycerides over 4 weeks as compared to the controls, and 8 weeks as compared to controls, losartan-, and CLA-treated, but not the telmisartan-treated, rats.
6. Telmisartan administration significantly reduced serum levels triglycerides over 8 weeks relative to controls.
7. No significant differences in serum HDL- and LDL-cholesterol concentrations were observed among the different dietary protocols over 8 weeks.
8. Losartan administration significantly reduced serum glucose levels over 4 weeks relative to the CLA-treated rats, and over 8 weeks relative to the controls.

9. CLA/telmisartan combination and the telmisartan administration significantly reduced serum glucose levels over 8 weeks relative to the controls.

10. Losartan, telmisartan, and the CLA + telmisartan administration significantly reduced systolic blood pressure as compared to the controls over 12 weeks. Telmisartan alone reduced SBP when compared to CLA and CLA/telmisartan protocols.

11. CLA/telmisartan co-therapy significantly reduced plasma insulin levels when compared to controls and losartan-treated, but not the CLA- and the telmisartan-treated, rats over 20 weeks.

12 Relative to the controls, pro-inflammatory cytokine IL1-α levels were slightly higher in telmisartan- and CLA-treated groups but lower in CLA + telmisartan group. Pro-inflammatory cytokines IL1-β and IL-6, and remarkably TNF-α, levels were higher in all dietary treatments.

13. Relative to the controls, IFN-γ levels were only higher in losartan- and telmisartan-treated groups whereas anti-atherogenic cytokines, IL-4 and IL-10, levels were increased in all dietary treatments, but more notably increased in CLA- and CLA + telmisartan-treated animals.

14. Plasma leptin levels were particularly higher in CLA-treated, but not other groups of, animals compared to the control group.

15. Rats on the CLA and telmisartan co-administration exhibited notable diminution in visceral adipose tissue size relative to animals in other protocols.

7.3.2 Study Conclusions

MetS remains an exciting and relevant research area. As a clinical entity, MetS will have to be targeted aggressively as a means of reducing the burden of CVD in industrialized countries. Although we do understand some of the molecular and physiologic mechanisms underlying MetS, the most appropriate and effective therapeutic approaches for the treatment of the syndrome and its associated features have yet to be identified. In that context, and based on the results of the present study, the use of CLA alone might not represent an appealing option. Alternatively, co-administration of CLA and telmisartan seems to present promising and attractive novel approach.

CLA has been shown to activate PPARγ in ZDF (Houseknecht et al. 1998) and PPARα in SD (Moya-Camarena et al. 1999a) rat models. In SD rats, CLA did show a trend toward, but did not significantly affect, regulation of PPARγ (Moya-Camarena et al. 1999b; Choi et al. 2004). On the other hand, a few studies in SD rats provided evidence that telmisartan appears to act as a partial agonist of PPARγ, and cause modest activation of PPARα (Benson et al. 2004; Schupp et al. 2004). Therefore, our study raises the possibility that the observed changes with CLA/telmisartan co-therapy on body weight, and glucose and lipid metabolism might be attributed, at least in part, to an increase in degrees of affinity and activation potentials of PPARα and/or PPARγ.

Many researches now believe that, for treatment or management of the MetS, the dual PPARα/PPARγ agonists are an attractive option because these

agonists have two separate targets — metabolic pathways in adipose tissue and liver. They improve both hyperglycaemia and atherogenic dyslipidaemia and may further reduce the inflammatory component of atherogenesis. By stimulating two nuclear receptors at a time, agonists of PPARα and PPARγ have a multiplicity of actions, which although could potentially increase the efficacy of therapy may also compound the side effects (Nissen et al. 2005). These side effects might not appear in the case of CLA/telmisartan owing to the fact that, at least, telmisartan is a partial, rather than full agonist of PPARs. Ultimately, the therapeutic potential of CLA plus telmisartan against individual components of MetS-associated CVD, although promising, has yet to be fully understood.

7.4 STRENGTHS AND LIMITATIONS OF THE STUDY

7.4.1 Strengths

Study design and length are among strengths of our study. We carried out the study for a relatively long period – 20 weeks – because MetS is a chronic condition that has been reported to increase with age.

Moreover, this study is related to human health and well-being. We believe that research on the effects of nutraceuticals (i.e. CLA) in human health, and understanding the biochemical and molecular mechanisms by which food affects health in order to authenticate new food products are important areas for research.

Another strength of the present study might lie in the fact that we used a normal animal model under conditions of high calorie diet. This would mimic the current pattern of Western lifestyle and would, in turn, offer better picture of the effect of our protocol if to be applied to human subjects.

One other strength is that we have been preparing fresh food each day for the animals during the experimental period. This would guarantee that there would be no, or very minimal, oxidation of the CLA in diets. Further, we measured food and water intakes daily during the course of the experiments. This allowed us to identify the approximate daily consumption of diet and drugs.

Finally, our novel notion of investigation of the combination effects of dietary agent (CLA) with the new ARB/PPARγ partial agonist telmisartan is an important part of the study.

7.4.2 Limitations

The present study, like most others, has also some limitations. The first limitation of this study is that we collected blood and analyzed serum metabolites only at baseline, wk 4, and wk 8, although the study was continued until wk 20. We did not collect blood at wk 12 and wk 16. The fact is that we started at wk 12 recording BP, a process that lasted for almost 4 weeks. The BP recording technique is a long and a stressful process for the animals, and should be performed in a calm situation. For that reason, we did not want to place the animals under more stress through blood collection

procedures. Nevertheless, we did collect blood by cardiac puncture, and obtained plasma at the end of the study for future analysis. Interrelated with the previous point, we did not measure animals' BP at baseline or during early weeks of the experimental period due to technical difficulties.

Because of the high expenses of the cytokine assay, we have pooled plasma samples before cytokine levels were measured. As a result, statistical interpretations for our observations could not be applied. We, nonetheless, did compare percentages of optical density data in treated animals and controls, which provided some evidence for an effect by the combination therapy on plasma cytokines.

Finally, it would be better if we would have measured plasma concentrations of the drugs and CLA to correlate the observed effects with plasma levels of these agents.

7.5 FUTURE DIRECTIONS

Now that we have demonstrated several different effects of CLA and telmisartan co-administration on a diet-induced MetS in a SD rat model, identifying the underlying mechanisms of action is warranted. To understand the mechanisms via which the combination of CLA and telmisartan improved body weight, lipid, and glucose and insulin metabolism, it may be beneficial that analysis of the following factors/parameters be considered in future studies:

1. Levels of PPARα and PPARγ mRNA in liver and adipose tissues.

2. Levels of mRNA for transcription factors other than the PPARs, NF-κB and the SREBPs (-1a, -1c, and -2).

3. Investigating the efficacy of specific isomers of CLA with telmisartan.

Moreover, determining the expression of some of the PPARγ candidate genes that are involved in adipocyte differentiation (i.e. C/EBPα and STAT1A), or glucose uptake (i.e. GLUT4, adiponectin, and resistin), as well as those involved in lipid metabolism (aP2, heparin-releasable LPL, and CD36) may well help offering conclusive answers. As well, investigating the plasma phopholipid and tissue FA profiles, and further studies in other animal models such as the PPARα- and PPARγ-null mice, or the ZDF rats will also provide additional supportive data.

8.0 REFERENCES

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