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RESEARCH ARTICLE

Hepatic steatosis by dietary-conjugated linoleic acid is accompanied by accumulation of diacylglycerol and increased membrane-associated protein kinase C ε in mice

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Scope: Conjugated linoleic acid reduces weight gain and adipose mass while inducing liver enlargement, hepatic steatosis, and insulin resistance in mice. The objective of this study was to determine if hepatic steatosis induced by conjugated linoleic acid would predict for hepatic diacylglycerol accumulation, increased membrane-associated protein kinase C ε, and hyperglycemia.

Methods and results: Six-wk-old C57Bl/6 male mice were fed a high-saturated fat diet for 3 wk and were then randomized to high-saturated fat diet with or without conjugated linoleic acid (1.5% wt). Following a 6-wk intervention, hepatic triacylglycerol, diacylglycerol, membrane-associated protein kinase C ε, and gluconeogenic gene expression were determined. Fasting glucose was determined at baseline and at the end of the study. Conjugated linoleic acid increased hepatic triacylglycerol and diacylglycerol concentration in association with increased membrane-associated protein kinase C ε. Diacylglycerol concentration proved to be a better predictor than triacylglycerol concentration for the change from baseline in fasting glucose concentration and final insulin concentration. The increase in diacylglycerol concentration was also associated with increased hepatic gluconeogenic gene expression in conjugated linoleic acid-treated animals.

Conclusion: Our data suggest that conjugated linoleic acid can initiate the pathophysiology responsible for hepatic insulin resistance. Additional studies are needed to determine if the accumulation of hepatic diacylglycerol by conjugated linoleic acid leads to hepatic insulin resistance.

Keywords:
Conjugated linoleic acid / Diacylglycerol / Hepatic steatosis / Insulin resistance / Protein kinase C ε

1 Introduction

Obesity is a major contributor to insulin resistance and the metabolic syndrome due to ectopic accumulation of lipids in nonadipose tissues, such as liver. Hepatic steatosis is classically defined as >5% liver weight as triacylglycerol (TG) and occurs when the rate of hepatic fatty acid uptake and de novo lipogenesis exceed hepatic fatty acid oxidation and TG export [1]. Hepatic steatosis plays an important role in the pathogenesis of hepatic insulin resistance and increased hepatic gluconeogenesis [2–4]. In particular, Samuel and colleagues propose that the accumulation of diacylglycerol

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Abbreviation: CLA, conjugated linoleic acid; **CON,** control; **DAG,** diacylglycerol; **FAS,** fatty acid synthase; **FAT/CD36,** fatty acid translocase; **G6Pase,** glucose-6-phosphatase; **PEPCK,** phosphoenolpyruvate carboxykinase; **PKCε,** protein kinase C ε; **PPARγ,** peroxisome proliferator-activated receptor γ; **SCD1,** stearoyl-CoA desaturase-1; **TG,** triacylglycerol

(DAG) along with TG leads to hepatic insulin resistance as characterized by blunted insulin signaling in the liver [5]. The accumulation of hepatic DAG activates protein kinase C ε (PKCε), leading to an inhibition of insulin signaling and increased gluconeogenesis [5, 6].

With the need to reduce obesity, numerous dietary supplements have been studied in regard to their ability to reduce weight and adiposity. Conjugated linoleic acid (CLA) has emerged as an effective weight loss agent in some rodent and human studies [7–10]. Twenty-eight isomers of CLA exist although the adipose-lowering effects are attributed to the trans-10, cis-12 isomer [11, 12]. CLA is found in ruminant dairy and meat but the trans-10, cis-12 isomer is primarily consumed as a mixed isomer weight-loss supplement. While reducing adipose mass, CLA paradoxically increases hepatic lipid deposition and causes insulin resistance in mice in a lipodystrophic manner [8, 13, 14]. Previous reports indicate that CLA increases transcription of genes involved in hepatic fatty acid uptake and lipogenesis [13, 15–18]. These findings led us to hypothesize that the exacerbation of hepatic steatosis by dietary CLA predicts for hepatic DAG accumulation, hepatic membrane-associated PKCε, and increased markers of hepatic insulin resistance relative to high-saturated fat diet alone.

2 Materials and methods

2.1 Experimental animals and diets

Six-wk-old C57Bl/6 male mice acclimatized for 3 wk while being fed a high-saturated fat diet (23.6% total fat by weight, Research Diets, New Brunswick, NJ) in an effort induce mild obesity as described previously [16] (Table 1). Mice were then randomly assigned to control (CON) or CLA dietary intervention ($n = 10$ mice per group) groups which is denoted as baseline (wk 0). The experimental intervention occurred over a 6-wk period with isocaloric diets (Table 2) containing 22.1% lard oil supplemented with 1.5% soybean oil (CON) or 1.5% CLA oil (Cognis, Cincinnati, OH). The trans-10, cis-12, and cis-9, trans-11 isomers accounted for 38.5 and 36.9%, respectively, as we have determined by gas chromatography [14] (Table 3). Mice had free access to food and water throughout the study. Following the intervention, animals were euthanized by isoflurane and tissues were immediately excised and flash-frozen with liquid nitrogen. Tissues were stored at −80°C for analyses. Procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of The Ohio State University.

2.2 Serum metabolites

Fasting blood was obtained for glucose analysis via retro-orbital bleed at baseline (wk 0) and by cardiac puncture at the end of the study (wk 6). Fasting glucose concentrations were determined by glucose monitoring system (One Touch, LifeScan, Milpitas, CA). Other serum analytes were reported previously [16] and are summarized in Table 1 for comparison purposes.

Table 1. Effects of 1.5% (wt) dietary CLA on food intake, body weight, tissues weights, and serum analytes

	CON	CLA
Animal data		
Food intake (g/wk/mouse)	28.9 ± 1.2	33.6 ± 0.7
Initial body weight (g)	22.7 ± 0.3	24.1 ± 0.4*
Final body weight (g)	29.9 ± 0.6	28.9 ± 0.6
Change in body weight (g)	7.2 ± 0.7	4.8 ± 0.3*
Tissue weights		
Liver (g)	1.05 ± 0.1	1.76 ± 0.1*
Epididymal adipose (g)	1.24 ± 0.1	0.26 ± 0.1*
Inguinal adipose (g)	0.59 ± 0.1	0.12 ± 0.1*
Tissue weight percentages		
Liver %	3.75 ± 0.1	6.26 ± 0.6*
Epididymal adipose %	4.65 ± 0.5	0.94 ± 0.2*
Inguinal adipose %	2.20 ± 0.3	0.25 ± 0.1*
Serum analytes		
Initial insulin (pg/mL)	302.8 ± 13.5	248.6 ± 23.1
Final insulin (pg/mL)	851 ± 176	2638 ± 323*
Triglyceride (mg/dL)	44.8 ± 4.2	19.9 ± 3.5*
Nonesterified fatty acid (mmol/L)	0.59 ± 0.11	0.44 ± 0.04*
Leptin (pg/mL)	7241 ± 1738	1069 ± 322*
Adiponectin (μg/mL)	13.6 ± 2.7	2.1 ± 0.3*

Data are mean ± SE of six to ten mice per treatment as reported previously [16]. Tissue weights were determined at the end of the study (wk 6). Initial insulin was determined at baseline (wk 0) and all other serum analytes were determined at the end of the study (wk 6). *$p < 0.05$.

2.3 Extraction of hepatic lipid

Liver samples (~100 mg) were homogenized on ice for 15 s per sample in 10 × (v/w) tissue lysis buffer containing 20 mM trizma base, 1% Triton X-100, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM $Na_4P_2O_7 \cdot 10H_2O$, and protease inhibitors. Lipids were extracted using the method of Folch et al. [19]. Lipid extracts were resuspended in hexane-methylene chloride-ethyl ether (94.5:5:0.5, v/v/v) in preparation for solid-phase chromatography.

2.4 Separation and quantification of hepatic TG and DAG

Following extraction, the samples were analyzed as described previously [20]. In brief, TG and DAG were separated using a diol-bonded-phase SPE column (Waters, Milford, MA) while under vacuum. Each sample was injected onto the column and TG was eluted with hexane-methylene chloride-ethyl

Table 2. Diet composition

	CON	CLA
Nutrient % (wt/wt)		
Protein	23.6	23.6
Carbohydrate	40.2	40.2
Fat	23.6	23.6
Ingredient (g)		
Casein, 80 Mesh	200	200
L-cystine	3	3
Corn starch	72.8	72.8
Maltodextrin 10	100	100
Sucrose	172.8	172.8
Lard	190.5	190.5
Soybean oil	13	–
CLA oil	–	13
Cellulose, BW200	50	50
Mineral mix	10	10
DiCalcium phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate, H_2O	16.5	16.5
Vitamin mix	10	10
Choline bitartrate	2	2

Table 3. Dietary oil fatty acid composition

		% Fatty acid	
		Soybean oil (CON)	CLA oil (CLA)
C16:0	Palmitic acid	10.4	4.2
C16:1n7	Palmitoleic acid	–	0.1
C18:0	Stearic acid	3.8	2.2
C18:1n9	Oleic acid	24.3	11.2
C18:2n6	Linoleic acid	53.5	1.5
C18:2-CLA	cis-9, trans-11 CLA	–	36.9
C18:2-CLA	trans-10, cis-12 CLA	–	38.5
C18:3n3	Linolenic acid or α-linolenic acid	7.8	0.1
C20:0	Arachidic acid	–	1.0
C20:1n9	Eicosenoic acid	–	1.6
C20:5n3	Eicosapentaenoic acid	–	0.2

ether (89:10:1, v/v/v). DAG was eluted with hexane-ethyl acetate (85:15, v/v). Following separation, the samples were dried under nitrogen gas and resuspended in 100 μL of *tert*-butanol, methanol, Triton X-100 (3:1:1, v/v/v). TG and DAG concentrations were measured using a spectrophotometric assay from Sigma (Triglyceride, Free-Glycerol reagents, St. Louis, MO) and are expressed as mg/g of tissue.

2.5 Subcellular localization of PKC ε

PKCε fractions were determined via a slightly modified protocol from the methods described previously [21]. Liver samples (~300 mg) were homogenized and lysed in 5 × (v/w) tissue lysis buffer A (50 mM Tris · HCl, pH 7.5; 1 mM EDTA; 1 mM EGTA; 1.5 mM $MgCl_2$; 250 mM sucrose; 1 mM Na_3VO_4; 1 mM NaF; protease inhibitor tablets). The homogenates were centrifuged at 100 000 × *g* for 60 min at 4°C. The supernatants were used as the cytosolic fractions. The pellet was then resuspended in buffer B (buffer A; 1% Triton X-100) and incubated for 15 min on ice. Following incubation, the samples were centrifuged at 20 000 × *g* for 20 min at 4°C. The supernatants were used as the solubilized membrane fractions. Protein content of cell lysates was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) in preparation for Western blotting.

2.6 Western blotting

Protein from membrane and cytosolic fractions, 20 and 40 μg respectively, was separated on an 8% SDS-PAGE and transferred onto nitrocellulose membranes (0.45 μm). MemCode staining (Pierce) was employed to show equal protein loading and transfer. Membranes were then blocked with 5% nonfat dry milk and incubated overnight with anti-PCKε primary antibody (Millipore, Billerica, MA). Membranes were then incubated with secondary antibody (HRP-linked anti-rabbit IgG, Cell Signaling, Danvers, MA) for 60 min and bands were visualized by chemiluminescence (Super Signal, Pierce) using Kodak Image Station 2000RT (Eastman Kodak, Rochester, NY). Membranes were then stripped and reprobed for β-actin (Cell Signaling). Relative densities were determined using Kodak 1-D 3.6 software and were normalized to β-actin. Membrane and cytosolic fractionation was validated by blotting for a membrane protein (insulin receptor β) and cytosolic protein (glyceraldehyde 3-phosphate dehydrogenase).

2.7 RT-PCR

Total RNA was extracted from liver samples using TriZol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA concentration was determined spectrophotometrically, and RNA quality was visualized on a 1% agarose gel. RNA was then reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). cDNA was amplified by real-time PCR with TaqMan Gene Expression Assays (Prism 7300 sequence detection system, Applied Biosystems) using predesigned and validated primers (FAM probes) under universal cycling conditions defined by Applied Biosystems. Target gene expression was normalized to the endogenous 18 s ribosomal RNA (VIC probes) amplified in the same reaction and expressed as $2^{-\Delta\Delta ct}$ relative to the CON group [22].

2.8 Statistical analyses

Comparison of dietary treatment effects was performed using Student's *t*-test (MINITAB 15, State College, PA). Pearson's rank correlation coefficient (MINITAB 15) was used to calculate the correlation coefficients between serum insulin, glucose, and liver TG and DAG concentrations. All data are presented as mean ± standard error. Differences were considered significant at $p < 0.05$.

3 Results

3.1 CLA increases fasting glucose and gluconeogenic enzyme expression

As previously reported, dietary CLA significantly reduced weight gain and adipose mass concomitant with liver enlargement and hyperinsulinemia [16] (Table 1). Despite these changes, average food intake was not different between CON (28.9 g/mouse/wk ± 1.2) and CLA (33.6 g/mouse/wk ± 0.7) treatments. The high-saturated fat CON diet increased fasting serum glucose concentrations over the 6-wk study. However, CLA treatment increased fasting serum glucose levels from baseline by nearly 85%, whereas the CON group increased by 49% during the same timeframe (Fig. 1A). In addition, the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were induced by CLA treatment by 73 and 33%, respectively (Fig. 1B).

3.2 CLA exacerbates hepatic steatosis and increases hepatic DAG

Relative to CON, CLA treatment increased hepatic lipid deposition by the end of the study. CLA treatment increased the percentage of hepatic lipid to nearly double that of the high-saturated fat CON diet. CLA treatment increased hepatic TG by 77% over CON and increased hepatic DAG by 169% over CON by the end of the study (Fig. 2A and B). Interestingly, hepatic DAG concentrations were shown to be considerably better predictors of the change in fasting serum glucose and final insulin concentrations when compared with TG (Fig. 2C–F). These correlations suggest that hepatic DAG, and not TG, plays a critical role in the development of CLA-induced fasting hyperglycemia and hyperinsulinemia.

3.3 CLA increases hepatic membrane-associated Pε

The previous reports have implicated a DAG-mediated activation of Pε in the development of hepatic insulin resistance [5, 6]. We sought to determine if the increased hepatic DAG observed with CLA treatment would also initiate Pε translocation to the membrane. Due to Pε, being translocated to the membrane upon activation [5, 21], we interpreted an increase in membrane to cytosolic ratio as a marker of activation. As shown in Fig. 3, CLA nearly doubled the membrane to cytosolic ratio of Pε by the end of the study.

4 Discussion

Obesity and obesity-related disorders remain paramount to the development of insulin resistance and the metabolic syndrome. Hepatic steatosis is highly prevalent in obese populations [1] and is likely involved in the initiation of insulin resistance, the metabolic syndrome, and type 2 diabetes [2–4]. High-fat feeding of rodents has established the role of hepatic DAG in the pathogenesis of hepatic insulin resistance and subsequent hepatic gluconeogenesis [5, 6]. CLA has received considerable attention due to its ability to reduce adiposity in a variety of species. However, CLA treatment also has deleterious effects that result in liver enlargement and increased hepatic lipid deposition. In the present study, we report, for the first time, that dietary CLA increases hepatic DAG, membrane-associated PKCε, and promotes the pathophysiology responsible for the development of hepatic insulin resistance.

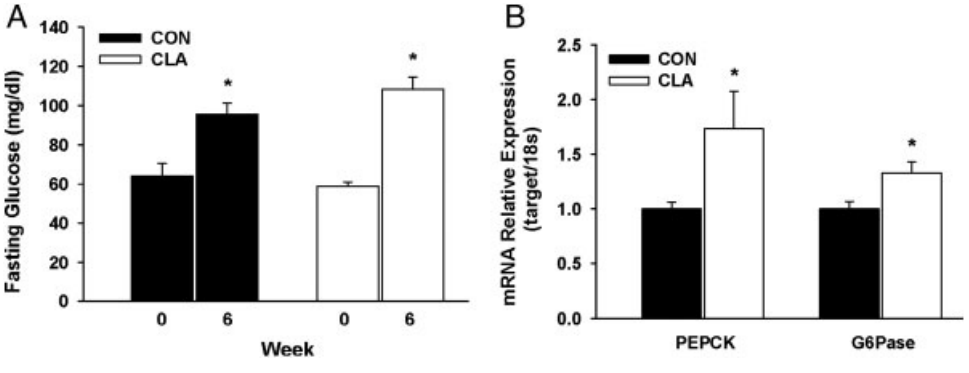

Figure 1. Effects of dietary CLA on fasting glucose and gluconeogenic gene expression. (A) Change in fasting glucose levels compared with baseline (wk 0) within each treatment group. (B) PEPCK and G6Pase mRNA levels in mice with or without dietary CLA for 6 wk. Data are mean ± SE of seven to ten mice per treatment. $^{*}p < 0.05$.

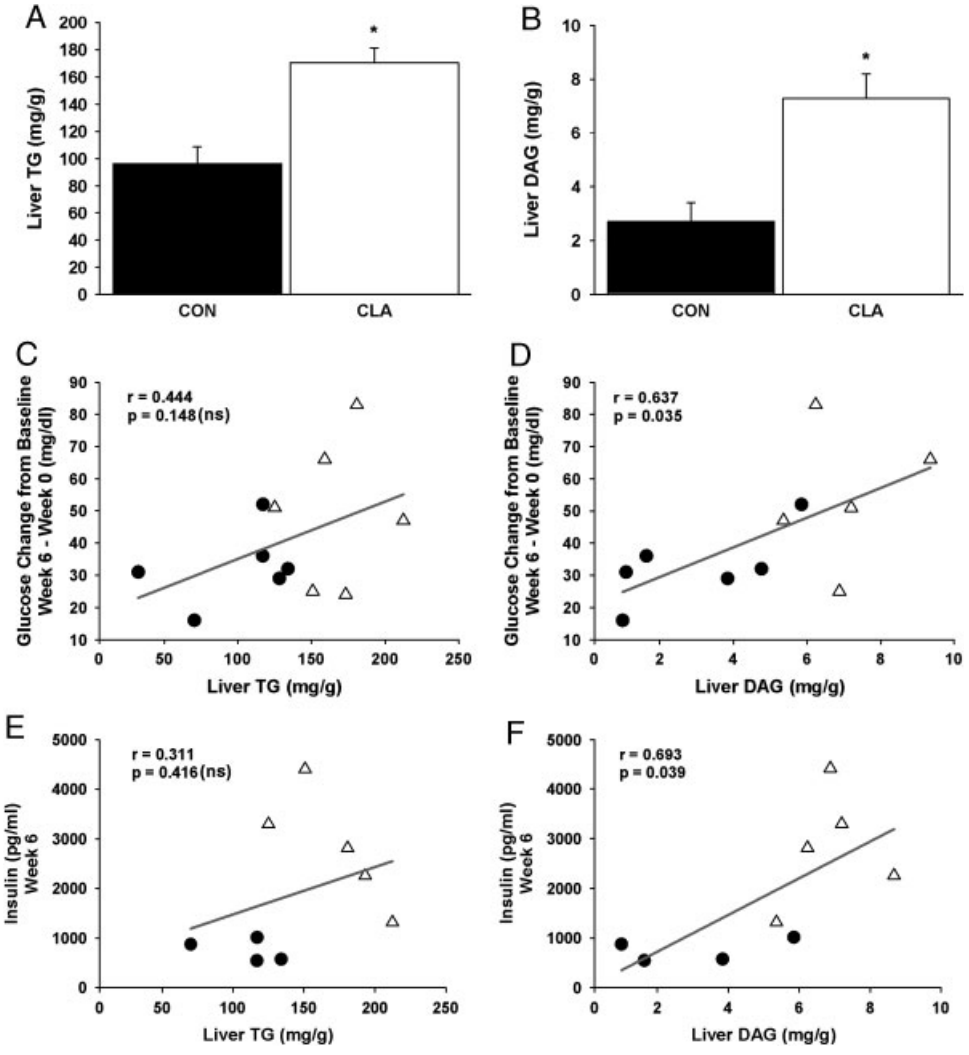

Figure 2. Effects of dietary CLA on hepatic lipid content and their correlation to change in glucose and final insulin. (A) Liver TG content, (B) liver DAG content, (C) correlation of TG content to change in fasting glucose concentration, (D) correlation of DAG to change in fasting glucose concentration, (E) correlation of TG content to final fasting insulin concentration, and (F) correlation of DAG content to final fasting insulin concentration. Data are mean ± SE of six to ten mice per treatment for (A and B). For (C–F), CON is represented by black circles (●) and CLA is represented by white triangles (Δ). $^{*}p < 0.05$ and (ns) represents not significant.

We previously reported that 1.5% dietary CLA (wt/wt) significantly reduced weight gain and adipose mass in male C57Bl6 mice fed a high-saturated fat diet [16]. These observations were accompanied by metabolic disorders including hepatomegaly, hyperinsulinemia, insulin resistance, and reductions in serum adipokines, leptin, and adiponectin. Our findings are analogous with other reports in which feeding-purified trans-10, cis-12 CLA, or mixed isomer CLA reduced fat mass while causing fatty liver and hyperinsulinemia [8, 13, 14]. The mechanism responsible for CLA-induced hepatic steatosis likely involves an increase in hepatic fatty acid uptake and lipogenesis. Clement et al. reported that 0.4% dietary CLA (wt/wt) increased the expression of adipocyte lipid-binding protein (ALBP, also known as aP2), fatty acid synthase (FAS), and fatty acid translocase (FAT/CD36) in the liver [13]. These modifications are likely the result of a concomitant induction of hepatic peroxisome proliferator-activated receptor γ (PPARγ) and sterol-responsive element-binding protein-1a (SREBP1a), which are involved in hepatic adipogenic [23] and lipogenic [24] programming, respectively. Takahashi et al. later confirmed increases in lipogenic programming with CLA treatment through increased expression and activity of acetyl-CoA carboxylase (ACC), ATP-citrate lyase (ACL), FAS, and stearoyl-CoA desaturase-1 (SCD1) [15]. These studies strongly suggest that CLA induces the expression of enzymes involved in fatty acid uptake, trafficking, and synthesis. We have also observed an increase in hepatic adipogenic and lipogenic programming in our current model. We previously reported an induction in hepatic PPARγ, FAS, FAT/CD36, and SCD1 with 1.5% dietary CLA (w/w) [16]. These findings led to the analysis of hepatic TG and DAG and their potential role in CLA-induced insulin resistance.

In the current study, 1.5% CLA increased hepatic TG content by 77% over high-saturated fat diet alone. This finding is in contrast to a report by Tsuboyama-Kasaoka et al., in which increased dietary fat diminished the lipodystrophic effects of CLA [25]. These differences could be attributed to their use of female mice or a lower concen-

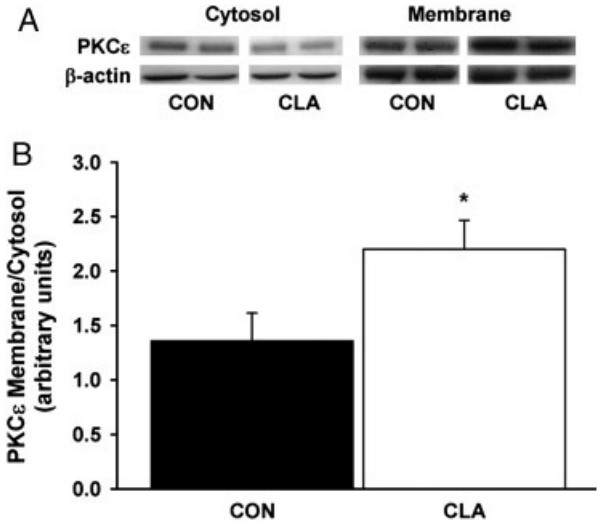

Figure 3. Effects of dietary CLA on hepatic PKCε translocation. (A) Representative blots showing membrane and cytosolic fraction of hepatic PKCε and β-actin from CON and CLA mice. (B) Data were quantified by densitometry and normalized to β-actin. Data are mean ± SE of four mice per treatment. $^{*}p < 0.05$.

tration of trans-10, cis-12 CLA. However, serum leptin concentrations were maintained in their study which is shown to diminish CLA-induced hepatic lipid deposition [26]. In addition to increased hepatic TG, we also report a 169% increase in hepatic DAG content with CLA treatment. Hepatic DAG has been implicated in hepatic insulin resistance and increased gluconeogenesis [5]. Interestingly, we report that hepatic DAG concentration may be predictive of the change in fasting serum glucose and final serum insulin concentrations. As shown in Fig. 1A, CLA treatment initiated a significant increase in fasting serum glucose over baseline values. The high-fat CON diet also increased fasting serum glucose levels from baseline although CLA treatment had an additive effect to the high-fat CON diet alone. These findings can be at least partially attributed to an increase in hepatic gluconeogenesis because fasting hyperglycemia is directly related to increased endogenous glucose production [27]. Additionally, CLA also induced the expression of hepatic PEPCK and G6Pase which are rate-limiting enzymatic mediators of gluconeogenesis. The significance of this finding is presently unknown because a recent report suggests that the induction of PEPCK and G6Pase is not required for increased hepatic gluconeogenesis in rats [28].

The mechanism by which DAG mediates insulin resistance of the liver is through the activation of Pε [5, 6]. PKCε is a prominent serine and threonine kinase that is known to be activated by DAG and has been shown to interfere with tyrosine phosphorylation [29]. Samuel and colleagues have demonstrated the association between PKCε activation and decreased insulin receptor substrate-2 (IRS2) tyrosine phosphorylation [5]. This finding was accompanied by increased gluconeogenesis which would likely lead to hyperglycemia over a longer study. The suppression of PKCε by antisense oligonucleotide restores hepatic insulin signaling despite the elevated levels of DAG [6]. In association with increased hepatic DAG, CLA feeding also increased membrane-associated PKCε in the current study (Fig. 3). We propose that this finding contributes to our observed increase in fasting glucose concentration with CLA feeding and likely is a contributing factor to CLA-induced insulin resistance.

Although we have evidence to suggest that the DAG-mediated pathway is involved in CLA-induced hyperinsulinemia and insulin resistance, the role of leptin and adiponectin cannot be ignored. Both adipokines have been reported to abrogate insulin resistance in obese and lipodystrophic models [30, 31]. Leptin secretion is proportional to adipose mass which explains the reason why CLA supplementation indirectly yet commonly reduces serum concentrations of leptin [32]. On the contrary, CLA appears to directly diminish adiponectin expression and assembly in adipocytes through PPARγ-dependent and PPARγ-independent mechanism [33]. Poirier et al. reported that CLA-induced hyperinsulinemia is closely associated with reductions in serum leptin and adiponectin [34]. We have previously reported that recombinant leptin injections or adiponectin recovery diminishes hyperinsulinemia and insulin resistance induced by CLA [26, 35]. The reductions in serum leptin and adiponectin [16] indirectly support the importance of adipokines in maintaining hepatic insulin sensitivity. It is likely that both a reduction in serum adipokines and the DAG-mediated mechanism are involved in CLA-induced metabolic abnormalities.

In summary, this study demonstrates that CLA-induced delipidation of adipose is accompanied by an increase in hepatic DAG and membrane-associated PKCε. Additionally, hepatic gluconeogenesis appears to be increased which could be partially responsible for hyperglycemia and hyperinsulinemia. Further studies, including hyperinsulinemic-euglycemic clamps, are required to determine if CLA-induced hepatic DAG accumulation causes hepatic insulin resistance and subsequently leads to peripheral insulin resistance.

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The authors have declared no conflict of interest.

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