Maintenance of adiponectin attenuates insulin resistance induced by dietary conjugated linoleic acid in mice

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Running Title: CLA and insulin resistance

# Authors contributed equally

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Abbreviations: Acetyl CoA oxidase (AOX), area under the curve (AUC), conjugated linoleic acid (CLA), fasting blood glucose (FBG), fatty acid synthase (FAS), fatty acid transporter (CD36), insulin resistance (IR), insulin tolerance test (ITT), non-esterified fatty acid (NEFA), rosiglitazone (ROSI), Triglyceride (TG), and tumor necrosis factor (TNF-α).
Abstract: Conjugated linoleic acid (CLA) causes insulin resistance and hepatic steatosis in conjunction with depletion of adipokines in some rodent models. Our objective was to determine if maintenance of adipokines mainly leptin and adiponectin by either removing CLA from diets or using an adiponectin enhancer, rosiglitazone (ROSI) could attenuate CLA induced insulin resistance. Male C57BL/6 mice were consecutively fed two experimental diets containing 1.5% CLA mixed isomer for four weeks followed by a diet without CLA for four weeks. CLA significantly depleted adiponectin, but not leptin and was accompanied by hepatic steatosis and insulin resistance. These effects were attenuated after switching mice to the diet without CLA along with restoration of adiponectin. To further elucidate the role of adiponectin in CLA mediated insulin resistance, ROSI was used in a subsequent study in male ob/ob mice fed either CON or CLA diet. ROSI maintained significantly higher adiponectin levels in CON and CLA fed mice and prevented depletion of epididymal adipose tissue and development of insulin resistance. In conclusion, we show that insulin resistance induced by CLA may be related more to adiponectin depletion than leptin and maintaining adiponectin levels alone either by removing CLA or using ROSI can attenuate these effects.

Keywords: Conjugated linoleic acid, hepatic steatosis, insulin resistance, adiponectin
Introduction

Type 2 diabetes is characterized by impaired glucose and lipid metabolism and is associated with obesity (1). Adipose tissue not only stores excess energy but also has important endocrine functions. Proteins secreted from the adipose tissue, known as adipokines, have important functions in regulating whole body metabolism (2). In particular, the adipokine adiponectin was identified in the adipose tissue and plasma of humans and rodents (3-6) and is inversely associated with obesity and type 2 diabetes (7;8). Administration of adiponectin attenuates insulin resistance (IR) by decreasing tissue triglyceride (TG) levels as a result of increased fatty acid oxidation (9;10). In addition, adiponectin lowers hyperglycemia by suppressing hepatic glucose production along with increasing glucose uptake by the skeletal muscle (8;9).

Conjugated linoleic acid (CLA) consists of positional and geometric isomers of octadecadienoate that are naturally found in foods such as beef, lamb, milk and other dairy products (11). It is well-established that \( c\text{9t11}-\text{CLA} \) and \( t\text{10c12}-\text{CLA} \) have unique effects on lipid metabolism (12) and it is the \( t\text{10c12}-\text{CLA} \) isomer that is mainly associated with decreases in body fat in experimental rodent models (13-16) as well as in some but not all human studies (17-19), independently of energy intake (20).

Feeding CLA to mice is associated with lipodystrophy and worsening of insulin sensitivity (16;21-24). These effects have been attributed to rapid and significant reduction of adipose tissue and a sharp decline in insulin sensitizing adipokines such as adiponectin and leptin (23). While feeding dietary CLA has been previously shown to deplete adipokines and cause hyperinsulinaemia (23), it is unknown whether removing CLA from the diet can restore the level of adipokines and attenuate insulin resistance.
showing that there is in fact, a causal link between adipokine depletion by feeding dietary CLA and development of hepatic steatosis and insulin resistance in mice. Further, dietary CLA has been shown to induce insulin resistance in \(ob/ob\) mice which lack functional leptin (16) raising the possibility that adipokines other than leptin may be more important in CLA mediated insulin resistance in mice. Thus, we further investigated the role of adiponectin in insulin resistance mediated by CLA in leptin deficient \(ob/ob\) mice. To this end, we used the peroxisome proliferator-activated receptor-gamma (PPAR-\(\gamma\)) agonist rosiglitazone (ROSI) in conjunction with CLA and hypothesized that maintaining serum adiponectin in \(ob/ob\) mice, which lack functional leptin attenuates the effects of CLA on insulin resistance and hyperglycemia.
Research Design and Methods

Materials

Diet components were purchased from Research Diets (New Brunswick, NJ) and Bio-Serv (Frenchtown, NJ) for studies 1 and 2 respectively. CLA mixed triglycerides (39.2% c9t11 and 38.5% t10c12 CLA) were obtained from Cognis (Cincinnati, OH). Rosiglitazone was obtained from Cayman Chemical (Ann Arbor, MI).

Animals

Eleven-week old male C57BL/6 mice and six week old male ob/ob mice were purchased from Harlan (Indianapolis, IN) and Charles River Laboratories, Inc. (Wilmington, MA) respectively. Mice were housed 4 per cage at 22 °C +/- 0.5 °C on a 12-hr day/night cycle. Mice received standard chow for one week while adjusting to their new environment. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Study 1

Depletion-repletion of Adipokines: To determine a role of adipokine depletion by CLA in the development of insulin resistance and hepatic steatosis, 12-week old male C57BL/6 (n=10) were consecutively fed two different experimental diets. For the first 4 weeks, mice were fed 1.5% CLA experimental diet, which contained 5% soybean oil plus 1.5% CLA triglyceride mix by weight for a total of 6.5% fat. This dose of CLA provided approximately 0.6% (by weight) each of the c9t11 and t10c12 CLA isomers. Supplementation with either 0.5% purified t10c12-CLA isomer or 1% CLA mixture has been shown to effectively reduce adipose tissue in mice and produce liver steatosis.
At the end of the first 4 week diet period, half of the mice were sacrificed and the remaining mice were switched to a diet without CLA (chow diet containing 4-5% total fat). All mice had free access to food and water. Body weights were measured at indicated time points.

**Study 2**

**Sustenance of adiponectin:** Six-week old male ob/ob mice were randomized by body weight and fed experimental diets containing 6.5% total fat for 4 weeks. The diets contained 6.5% soybean oil (CON diet, n=8) by weight. Additionally, ten mice were maintained on CON diet for the first 2 weeks following which six mice were switched to the CLA diet and four mice were continued on the CON diet for the last two weeks of study period. During the last two weeks mice received daily intra-peritoneal (IP) injections of PBS (vehicle control-DMSO 10% and PBS solution 90%) or 10mg/kgBwt/day ROSI (25;26). Body weights were measured weekly.

**Insulin tolerance test and Fasting blood glucose (FBG)**

Insulin sensitivity was determined for studies 1 and 2 using an insulin tolerance test at indicated times. Mice were fasted overnight and received intraperitoneal injections of insulin, Humulin R (Eli lily Inc) at doses of 0.75U/kg body weight for C57BL/6 and 1.5U/kg body weight for ob/ob mice. Insulin stimulated glucose clearance was determined by tail vein bleeding at 0, 15, 30, 45, 60, 90 and 120 min after insulin injection. Insulin sensitivity was determined by calculating the areas between the curves and individual baselines were used to normalize data. FBG levels were measured at baseline, two weeks and four weeks for study 2. Mice were fasted overnight for 12 hrs and tail vein blood was used to analyze FBG using a One Touch Basic glucose analyzer (Lifescan, Milpitas, CA).
Necropsy (study 1 and 2)

In order to avoid the effects of injected insulin on gene expression, mice were anesthetized by isoflurane in the fasted state 2 days after the insulin tolerance test. Blood was collected by heart puncture, centrifuged at 1500 x g at 4°C to isolate serum and stored at -80°C for hormone and metabolite analyses. Liver, epididymal adipose, and gastrocnemius muscle tissues were weighed, snap-frozen in liquid nitrogen, and stored at -80°C for further analysis.

Serum hormone and metabolite determination

Time course depletion-repletion of adipokines from study 1 were determined using 4-hr fasted serum from retro-orbital eye bleeds at the indicated time points. Mice were anesthetized using isoflurane. Additionally, fasted serum insulin, adiponectin and resistin levels from study 2 were determined using ELISA’s (LINCO Research, St. Charles, MO). Fasting serum triglycerides and non-esterified fatty acids (NEFA) from study 2 were measured using spectrophotometric assays from Sigma (St. Louis, MO) and Wako Chemicals, (Richmond, VA) respectively.

Tissue TG analysis (study 1 and 2)

Liver and muscle tissues were homogenized and lysed in 10x Tris (w/v) buffer containing 20mM trizma base, 1% trition-X100, 50mM NaCl, 250mM sucrose, 50mM NaF, 5mM Na₄P₂O₇·10H₂O and protease inhibitors. TG were extracted with 2:1(v/v) chloroform:methanol, final extracts were dissolved in 3:1:1 (v/v/v) tert-butanol:methanol:triton X-100 (27) and TG were quantitatively measured with an enzymatic colorimetric kit (Sigma). Values were expressed as percentage tissue weight.
Real-time RT PCR (study 2)

Sections from liver and muscle tissue were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) and RNA was isolated using the manufacturer’s protocol. RNA from adipose tissue was isolated using the RNeasy lipid extraction kit (Qiagen, Valencia, CA). RNA was diluted in RNase-free water and quantified by spectrophotometry. RNA integrity was assessed by electrophoresis using agarose gel and ethidium bromide staining. The first transcripts were reverse transcribed using reverse transcriptase (Invitrogen) and cDNA was amplified using real-time PCR with FAM labeled TaqMan gene expression assays (Applied Biosystems, Foster City, CA). In short, 5 ng of the reverse transcription reaction was amplified in a total reaction volume of 25µl using pre-designed and validated primers for liver fatty acid synthase (FAS), fatty acid transporter (CD36), and acetyl CoA oxidase (AOX), and tumor necrosis factor (TNF-α) using universal cycling conditions. Target gene expression was normalized to Vic labeled18s, which was used as an endogenous control and amplified in the same reaction as the target gene.

Statistical analysis

All data are presented as mean ± SE. Data were analyzed using MINITAB (version 14, PA). Data from study 2 were analyzed by one-way ANOVA. Post-hoc analysis was performed using Tukey’s test. Other comparisons were analyzed by Student’s t test as appropriate. Weight gain and serum adipokine concentrations over time were analyzed by repeated measures ANOVA using Statistical Analysis System (version 9.1, Cary, NC). Differences were considered significant at P < 0.05.
Results

Study 1

**Body weights and organ weights:** Body weights and weight gain were significantly reduced in C57BL/6 mice after 4 weeks of supplementation with dietary CLA (Table 1 and Figure 1A). Supplementation with dietary CLA significantly decreased epididymal adipose mass and increased liver weight. When CLA was removed from the diets, body weight and adipose tissue mass increased significantly (Table 1). Concomitant with increased body weight and adipose tissue weight, liver weights significantly decreased after four weeks on the diet without CLA (Table 1).

**Serum metabolites:** Levels of adiponectin decreased over time in C57BL/6 mice on the diet containing CLA. Significant differences were observed at day 6 and adiponectin levels continued to decrease over-time (Figure 2B). Switching mice to the diet without CLA significantly increased adiponectin levels however, levels remained significantly lower (50% of baseline) than baseline in C57BL/6 mice. In contrast, leptin levels were less responsive to dietary CLA and were not significantly different compared to baseline (Figure 2A).

**Insulin tolerance:** Insulin sensitivity was significantly worsened in C57BL/6 mice after 4 weeks on the CLA diet (AUC, Table 1 and Figure 3A). Switching mice to the diet without CLA significantly improved insulin sensitivity. The improvement in insulin sensitivity was significant after two weeks following the switch to the diet without CLA.

**Liver and Muscle TG:** Corresponding to increased liver weights, 4 weeks of feeding dietary CLA significantly increased hepatic TG in C57BL/6 mice. Switching to
the diet without CLA for 4 weeks significantly attenuated hepatic steatosis (Table 1). Muscle TG levels were not significantly different between the two diet groups (data not shown).

Study 2:

**Body weights and organ weights:** ROSI administration for two weeks prevented weight loss in male *ob/ob* mice fed dietary CLA and weight gain was comparable to CON-PBS mice in both ROSI treated groups (Figure 1B). Epididymal adipose mass was also not significantly different in the CLA-ROSI group compared to the CON-ROSI and CON-PBS group (Table 2).

**Serum metabolites:** Two weeks of treatment with ROSI not only prevented increases in both glucose and insulin in mice fed dietary CLA (CLA-ROSI vs. CON-ROSI and CON-PBS) but also significantly decreased FBG levels compared to CON-PBS mice (Table 2). ROSI administration significantly increased adiponectin levels in mice fed the CON or CLA diet compared to mice fed the CON-PBS diet (Table 2). Serum resistin levels were significantly higher in CON-PBS mice compared to CON-ROSI mice however; ROSI administration did not have an effect on resistin levels in mice fed CLA. Further, ROSI administration significantly decreased serum levels of TG and NEFA in both CON- and CLA-fed mice compared to CON-PBS group (Table 2).

**Insulin tolerance:** Compared to CON-PBS group, ROSI significantly improved insulin sensitivity in both CON- and CLA-fed *ob/ob* mice and CLA-ROSI fed mice had insulin sensitivity comparable to CON-ROSI mice (AUC; Table 2 and Figure 3B).
Liver and Muscle TG: While ROSI treatment did not decrease hepatic lipids in CON fed mice, interestingly, there was a significant reduction in liver TG in the CLA-ROSI group compared to CON-PBS and CON-ROSI groups after two weeks of ROSI treatment (Table 2). There was no effect of diet or treatment on muscle TG in ob/ob mice.

Liver mRNA expression: Because the combination of CLA with ROSI had significantly lower liver TG, we measured mRNA levels of genes indicative of lipid oxidation and lipid synthesis in the liver. ROSI treatment significantly increased mRNA level of liver AOX in both CON- and CLA-fed mice compared to CON-PBS mice (Figure 4A). There were no significant differences in the mRNA levels of peroxisome proliferator activated receptor (PPAR-α) and carnitine palmitoyl transferase (CPT-1) between the groups (data not shown). While ROSI treatment significantly increased mRNA levels of FAS in the CON fed mice, interestingly, the combination of CLA with ROSI had significantly lower levels of FAS mRNA comparable to CON-PBS mice (Figure 4B).

Muscle mRNA expression: Although muscle lipid content did not differ between diet groups, we measured mRNA levels of fatty acid transporter - CD 36 and AOX that have been previously shown to be up-regulated by adiponectin and have important functions in lipid metabolism (10). ROSI treatment significantly increased CD36 and AOX mRNA levels in mice fed dietary CLA (Figure 4C-D) compared to mice fed the CON diet.

Adipose mRNA expression: ROSI administration significantly decreased TNF-α mRNA levels in both CON- and CLA-fed mice compared to PBS injected mice fed the CON diet (Figure 4E).
Discussion

The effects of CLA on insulin sensitivity are controversial and vary depending on the species and level of dietary fat. Preliminary data from male and female \textit{ob/ob} mice showed that feeding dietary CLA (1.5% mixed isomer) as a part of 6.5% total fat diet for four weeks resulted in a significant decrease in body weight gain and adiposity along with an increase in hepatic steatosis (data not shown). The decrease in body weight measurements was significant by day seven in mice fed dietary CLA (data not shown). Along with decrease in weight gain, dietary CLA feeding resulted in a significant decrease in serum adiponectin and an increase in FBG levels by two weeks (data not shown). Serum levels of resistin were not increased by dietary CLA in \textit{ob/ob} mice after four weeks. Further, adipose mRNA levels of inflammatory cytokines e.g. TNF-\textit{\alpha} and IL-6 were also not significantly different between CON and CLA groups after four weeks (data not shown).

In the present study, we show that CLA causes rapid changes in weight and adiposity in mice. The depletion in adipose tissue is accompanied by worsened insulin sensitivity and development of hepatomegaly (likely attributable, in part, to increased hepatic lipids). These results are in accordance with other studies using both mixed isomers of CLA as well as the \textit{t10c12} isomer alone (16;21-24). Previously, it was shown that CLA causes a time course depletion of adiponectin and leptin and is associated with the development of insulin resistance in female C57BL/6J mice (23). Here, we show for the first time that removing CLA from the diet results in reversal of depressed adipokines, insulin resistance, and hepatic steatosis.
In study 1, the level of adiponectin was significantly depleted at six days in male C57BL/6 mice supplemented with dietary CLA. These results are in accordance with a study conducted by Poirier et al (23). However, in contrast to their study, in the present study, using comparable levels of dietary CLA, leptin levels were not significantly lower than baseline. These differences may be due to differences in gender in the two studies. Associated with the depletion of adiponectin, there was a significant decrease in phosphorylation of key mediators of glucose and lipid metabolism pathways such as AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in the livers of C57BL/6 mice and levels of these phospho-proteins were restored by removing CLA from the diet (data not shown). The observed decrease in phosphorylation state of AMPK and ACC may be indicative of decreased lipid oxidation in the liver and impaired insulin responsiveness (9;28-30). Switching mice to the diet without CLA resulted in a significant improvement in serum adiponectin levels as early as seven days. Of interest here, is that while insulin resistance and hepatic TG were significantly attenuated when CLA was removed from the diet, adiponectin levels were only partially restored suggesting that the presence of leptin may compensate for insufficient adiponectin levels and the overall adipokine status may influence the response to dietary CLA in different animal models. The concomitant repletion of adipokines and reversal of insulin resistance by removing CLA from the diets is novel and further supports a strong association between the dysregulation of adipokine synthesis by dietary CLA and worsening of insulin sensitivity in mice.

Previously, it was demonstrated that leptin infusion into C57BL/6 mice reverses insulin resistance and hepatic steatosis when adipose stores are not completely ablated (21). However, leptin has no effect when infused into A-ZIP/F-1 fatless mice (31). These data suggest that leptin insufficiency alone is not the principal cause for
lipodystrophy-associated insulin resistance caused by CLA (31). The effects of CLA on insulin resistance and hepatic steatosis have been previously demonstrated in ob/ob mice by Roche et al. (16). Preliminary findings from our study are in agreement with the study conducted by Roche et al. In addition, we measured serum adiponectin levels over time in female ob/ob mice fed CLA and found that levels were significantly and maximally depressed by two weeks (data not shown). Further, mice developed significant hepatic steatosis and insulin resistance along with the depletion of adiponectin. Because ob/ob mice lack functional leptin, these data in conjunction with our data from study 1 suggest that the worsening of insulin resistance caused by dietary CLA may be more strongly associated with depletion of adiponectin or overall adipokine status than leptin alone. To further examine the role of adiponectin in CLA induced insulin resistance, we injected leptin deficient male ob/ob mice with an adiponectin enhancer; ROSI for two weeks.

ROSI treatment significantly increased serum concentrations of adiponectin in mice fed either the CON or CLA diet compared to CON-PBS treated mice. These effects of ROSI on serum adiponectin are consistent with other studies (97, 87 120). Because higher circulating levels of serum resistin are often associated with insulin resistance and diabetes in rodents (32), we measured serum levels of resistin. Preliminary data from male ob/ob mice fed dietary CLA for four weeks did not have significantly increased resistin levels compared to CON-PBS mice (data not shown). Further, ROSI treatment did not have an effect on serum resistin levels in ob/ob mice fed dietary CLA. The lack of effect of ROSI on serum resistin levels in CLA fed mice further demonstrates that CLA mediated insulin resistance is related more to the depletion of adiponectin. The increase in serum adiponectin in the CLA-fed mice was accompanied by significantly lower levels of fasting glucose, insulin, insulin sensitivity, serum TG, and
NEFA similar to CON-ROSI treated mice. Co-treatment of CLA with ROSI for two weeks prevented lipodystrophy and associated increases in serum levels of glucose and insulin that are usually associated with supplementation of dietary CLA in mice (21-24). Additionally, ROSI treatment also prevented significant body weight loss which is often reported with treatment of CLA in mice. This may be attributed to maintenance of adipose mass as ROSI, a PPAR-γ agonist, often increases adipose mass (25;26). These data suggest that when adipose mass and adiponectin levels are maintained, the lipodystrophic effects associated with dietary CLA supplementation are attenuated.

In the present study, increased adiponectin levels were associated with higher levels of hepatic AOX mRNA in CON- and CLA-fed mice but only CLA-ROSI group had significantly lower hepatic FAS mRNA levels and hepatic TG. To our knowledge, the interactive effect of the combination of CLA and ROSI on hepatic TG is novel and suggests a complementary effect of these two agents for restoring normal lipid levels in the liver. Further, there were significant increases in the levels of the lipid transporter CD36 and lipid oxidative enzyme, AOX in the muscle of the CLA-ROSI group. Administration of adiponectin has similar effects on CD36 and AOX mRNA in mice fed high fat diets (10), suggesting that at least a part of effects of ROSI are mediated through adiponectin. Although it is unclear why the combination of CLA with ROSI has additional effects in the liver and muscle, it may be due to increased adiponectin levels with ROSI administration. It is possible that in the presence of adequate adiponectin, CLA increases lipid utilization and decreases lipid accumulation in tissues similar to observations from rat studies (33-35).

It has been shown previously that supplementation with dietary CLA significantly decreases adipokines and induces hyperinsulinaemia in female C57BL/6J mice by six
days (23). In the present study, ROSI was administered for two weeks. In this short term treatment, ROSI prevented CLA induced insulin resistance by maintaining adequate adiponectin levels in ob/ob mice. While we cannot speculate the effects of ROSI if treatment had been prolonged in this model, these results are in accordance with a recent study conducted in C57BL/6 mice (data submitted M.A. Belury) that were administered the combination of CLA-ROSI for six weeks.

Because other adipokines secreted from the adipose tissue such as TNF-α, IL-6 and resistin are known to modulate insulin sensitivity in addition to leptin and adiponectin, the overall adipokine status and not any particular adipokine alone may influence CLA’s effects on insulin resistance and hepatic steatosis. It was recently shown that short term t10c12-CLA administration induced adipose IL-6 and TNF-α mRNA without affecting serum levels (36). In contrast to these findings, preliminary data from male ob/ob mice fed mixed isomers of CLA for four weeks did not have significantly higher mRNA levels of TNF-α and IL-6 compared to CON mice (data not shown). Thus, while in the present study, adiponectin alone seems to be more important than other adipokines in insulin resistance mediated by CLA, future studies using adiponectin deficient mice are necessary to make conclusions regarding the relative importance of this adipokine. ROSI treatment significantly decreased adipose tissue TNF-α mRNA levels in both CON- as well as CLA- fed mice compared to CON-PBS treated mice. These data are in accordance with previous reports (37;38). Because ROSI is a thiazolidinedione known to improve insulin sensitivity possibly through multiple pathways, the increased synthesis of adiponectin is not the only possible explanation for our findings. In fact, ROSI may have additional effects in the adipose tissue and may decrease insulin resistance mediated by CLA by directly or indirectly modulating inflammatory cytokines such as TNF-α and IL-6.
In conclusion, we show that removing CLA from the diet of mice reverses insulin resistance and restores adiponectin establishing a link between depletion of this adipokine and development of hepatic steatosis and insulin resistance by dietary CLA in mice. We further show that in the absence of significant changes in leptin levels (male C57BL/6 mice fed dietary CLA) or in mice lacking functional leptin (male ob/ob mice), adiponectin depletion alone results in worsened insulin sensitivity by CLA. In addition, restoration of adiponectin (by either removal of CLA from diet or administering ROSI) is sufficient for reversal of these effects. Thus, in the present study, we show that adiponectin is an important factor responsible for insulin resistance caused by CLA provided as mixed isomer oil at 1.5 wt% of the diet. We based our dose (1.5wt% CLA equaling approximately 0.6wt% t10c12-CLA) on studies by others showing this dose to be effective for adipose suppression in mice. We observed that C57Bl/6 mice weighing an average ~30 g, ate ~ 2.0 g diet per day (and ob/ob mice weighing ~ 45 g, ate ~ 5.0 g diet per day). If this were translated to a human of ~ 55 kg, our dose of CLA could equal ~ 44 g CLA (or 21.2 g of t10-CLA). With this estimate, it seems an unrealistic choice for humans to use and achieve such a rapid loss of body fat with foods at this time. However, there are other factors to consider between species (mice vs. humans) including rate of metabolism of CLA isomers, rapidity of adipose tissue metabolism and specific to this design, differences in adipose catabolism. Nevertheless, we consider these pre-clinical studies to be important for improving our understanding of mechanisms of anti-adipose effects of CLA.
Acknowledgements

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Reference List


Figure Legends

Figure 1: Effect of dietary CLA on weight gain from study 1 and 2. (A) Male C57BL/6 mice were fed a diet containing 1.5% CLA (+CLA, n=10) for four weeks followed by four weeks without CLA (-CLA, n=5) * P<0.05 vs. baseline. § P<0.05 vs. last time point on the diet containing CLA. (B) Male ob/ob mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either PBS or ROSI for two weeks by IP injection daily. CON-PBS (n=8, closed circles), CON-ROSI (n=4, open circles), CLA-ROSI (n=6, closed triangles). * P<0.05.

Figure 2: Effect of CLA on time course depletion/repletion of adipokines from study 1. Male C57BL/6 mice were fed a diet containing 1.5% CLA (+CLA, n=10) for four weeks followed by four weeks without CLA (-CLA, n=5). (A) Serum leptin in male C57BL/6 mice; (B) serum adiponectin in male C57BL/6 mice. Adipokine concentrations were determined at indicated times. * P<0.05 vs. baseline, § P<0.05 vs. last time point on the diet containing CLA.

Figure 3: Effect of dietary CLA on insulin sensitivity. (A) Male C57BL/6 mice from study 1. Insulin sensitivity was measured at baseline (n=10, closed circles), after four weeks on dietary CLA (+CLA, n=10, open circles) and after two weeks on diet without CLA (-CLA, n=5, closed triangles); (B) male ob/ob mice from study 2. Insulin sensitivity was measured after two weeks on either control (CON) or CLA-supplemented (CLA) diets and injected with PBS or ROSI. CON-PBS (n=8, closed circles), CON-ROSI (n=4, open circles), CLA-ROSI (n=6, closed triangles). Significance was determined using AUC (Tables 1 and 2).
Figure 4: Effect of CLA on mRNA expression of genes involved in lipid metabolism from study 2. Male ob/ob mice were fed either control (CON) or CLA-supplemented (CLA) diets and received either PBS or ROSI for two weeks by IP injection daily. CON-PBS (n=8), CON-ROSI (n=4), CLA-ROSI (n=6) (A) hepatic AOX mRNA; (B) hepatic FAS mRNA; (C) muscle CD36; (D) muscle AOX; (E) epididymal adipose TNF-α. Vertical bars represent mean ± SE, * P<0.05.
Table 1: Body weights and organ weights—Study 1

<table>
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<th>Baseline</th>
<th>+CLA</th>
<th>-CLA</th>
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<tr>
<td>Body weight (g)</td>
<td>28.08 ± 0.73</td>
<td>27.211 ± 0.90 *</td>
<td>30.33 ± 1.60 §</td>
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<tr>
<td>Liver weight (% Bwt)</td>
<td>--</td>
<td>7.56 ± 0.83</td>
<td>4.28 ± 0.08 §</td>
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<td>Epididymal Adipose (g)</td>
<td>--</td>
<td>0.233 ± 0.03</td>
<td>0.38 ± 0.01 §</td>
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<td>Insulin stimulated glucose uptake (AUC)</td>
<td>-6272.14 ± 793.51</td>
<td>-1864.69 ± 581.99 *</td>
<td>-4735.00 ± 514.13 §</td>
</tr>
<tr>
<td>Liver TG (% Liver weight)</td>
<td>--</td>
<td>12.4089 ± 0.4968</td>
<td>2.8534 ± 1.1637 §</td>
</tr>
</tbody>
</table>

Body and organ weights of male C57BL/6 mice at baseline (n=10), after four weeks of feeding diet with +CLA (n=5) and after an additional four weeks of feeding diet without -CLA (n=5).

Values represent mean ± SE. Superscripts represent significant differences between treatments.

* P<0.05 vs. baseline, § P<0.05 vs. +CLA.
Male ob/ob mice were fed experimental diets containing 6.5% soybean oil (CON diet, n=8) for four weeks. Additionally, ten mice were maintained on CON diet for the first 2 weeks following which six mice were switched to the CLA diet containing 5% soybean oil plus 1.5% CLA mixed TG and four mice were continued on the CON diet for the last two weeks of study period and injected with either PBS (CON-PBS) or ROSI (CON-ROSI and CLA-ROSI). Values represent mean ± SE. Superscripts represent significant differences between treatments. Differences between means was calculated using one way ANOVA, values were considered significant at P<0.05.

Table 2: Body weights, organ weights and serum metabolites – Study 2

<table>
<thead>
<tr>
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<th>CON-PBS</th>
<th>CON-ROSI</th>
<th>CLA-ROSI</th>
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<tr>
<td>Final body weight (g)</td>
<td>42.20 ± 1.5</td>
<td>47.58 ± 2.12</td>
<td>46.5 ± 1.73</td>
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<td>Liver weight (% Bwt)</td>
<td>6.50 ± 0.35 a</td>
<td>6.91 ± 0.49 ab</td>
<td>8.35 ± 0.41 b</td>
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<td>Epididymal adipose (g)</td>
<td>2.96 ± 0.18</td>
<td>3.00 ± 0.27</td>
<td>3.15 ± 0.22</td>
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<td>Serum Insulin (pg/ml)</td>
<td>3099.64 ± 440.20 a</td>
<td>2180.24 ± 525.05 ab</td>
<td>1015.95 ± 80.95 b</td>
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<td>Resistin (ng/ml)</td>
<td>22.14 ± 1.02 a</td>
<td>14.72 ± 1.35 b</td>
<td>20.31 ± 1.54 ab</td>
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<td>Adiponectin (ng/ml)</td>
<td>10751.00 ± 1102.9 a</td>
<td>55996.00 ± 1459.0 b</td>
<td>56023 ± 1459.0 b</td>
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<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>6.82 ± 0.66 a</td>
<td>3.93 ± 0.98 b</td>
<td>4.70 ± 0.80 b</td>
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<td>Insulin stimulated glucose uptake (AUC)</td>
<td>-1735 ± 1144.29 a</td>
<td>-6760.00 ± 962.85 b</td>
<td>-5576.25 ± 920.97 b</td>
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<td>Serum TG (mg/dl)</td>
<td>88.13 ± 11.85 a</td>
<td>33.89 ± 14.52 b</td>
<td>44.27 ± 11.85 b</td>
</tr>
<tr>
<td>NEFA (mEq/L)</td>
<td>0.90 ± 0.09 a</td>
<td>0.40 ± 0.12 b</td>
<td>0.51 ± 0.09 b</td>
</tr>
<tr>
<td>Liver TG (% Liver weight)</td>
<td>18.1527 ± 1.9909 a</td>
<td>18.2921 ± 2.3035 a</td>
<td>10.2850 ± 0.5803 b</td>
</tr>
</tbody>
</table>
Figure 1

A

B

Weight Gain (g)

Day

Weight Gain (g)

Day

+ CLA

- CLA

ROS1

Downloaded from www.jlr.org by guest on September 9, 2017
Figure 2

A

Serum Leptin (ng/ml)

Day

0 10 20 30 40 50 60 70

+CLA  -CLA

B

Serum adiponectin (ng/ml)

Day

0 10 20 30 40 50 60
Figure 3

A

![Graph A](image)

B

![Graph B](image)
Figure 4

A

Ratio (AOX mRNA/18sRNA)

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON-PBS</td>
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</tr>
<tr>
<td>CON-ROSI</td>
<td>2.50</td>
</tr>
<tr>
<td>CLA-ROSI</td>
<td>2.50</td>
</tr>
</tbody>
</table>

B

Ratio (FAS mRNA/18sRNA)

<table>
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<th>Ratio</th>
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</thead>
<tbody>
<tr>
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<td>1.00</td>
</tr>
<tr>
<td>CON-ROSI</td>
<td>2.50</td>
</tr>
<tr>
<td>CLA-ROSI</td>
<td>1.50</td>
</tr>
</tbody>
</table>

C

Ratio (CD36 mRNA/18s mRNA)

<table>
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<th>Ratio</th>
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<tbody>
<tr>
<td>CON-PBS</td>
<td>1.00</td>
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<tr>
<td>CON-ROSI</td>
<td>2.00</td>
</tr>
<tr>
<td>CLA-ROSI</td>
<td>3.00</td>
</tr>
</tbody>
</table>