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# Conjugated nonadecadienoic acid is more potent than conjugated linoleic acid on body fat reduction

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**Abstract**

Conjugated linoleic acid (CLA) has shown a number of health benefits, particularly on controlling body fat while improving lean mass. As one of CLA cognates, conjugated nonadecadienoic acid (CNA, 19-carbon conjugated fatty acid) has been previously reported to have greater efficacy on body fat control. In this report, we compared the efficacy of dietary CLA and CNA on body fat regulation and also compared the mechanism of body fat control using a mouse model. Effects of 0.1% dietary CNA on body fat reduction were comparable to that of 0.5% dietary CLA. The mechanisms of dietary CNA on body fat control were similar to those of CLA: increased energy expenditure and increased fatty acid β-oxidation. Dietary CNA, but not CLA, also improved expression of hormone-sensitive lipase from white adipose tissue, and this may help explain how CNA has better efficacy on body fat control than CLA. Dietary CNA had similar effects as CLA on liver weights; however, unlike CLA, CNA improved glucose tolerance. Thus, CNA has potential to be used as a pharmacological agent to assist current efforts to reduce obesity with less adverse effects than CLA.


*Keywords:* CLA; CNA; Fat; Conjugated linoleic acid; Conjugated nonadecadienoic acid

## 1. Introduction

Conjugated linoleic acid (CLA) has received considerable attention due to potentially beneficial health benefits such as prevention of cancer and cardiovascular diseases, modulation of immune and inflammatory responses, growth promotion in young animals, and most importantly reduction of body fat while improving lean body mass [1,2]. This variety of biological activities by a relatively simple structured fatty acid may be explained by the fact that CLA is a mixture of isomers, in particular the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 as two major isomers. While both isomers are equally effective with regard to most anticancer activity, these two isomers have shown distinctive activities in other effects. The many physiological effects that are reported for CLA appear to be the result of multiple interactions of these two biologically active CLA isomers [1].

Structure–activity relationship study with CLA indicated that CLA's conjugated double bonds are required for its biological function [3-5]. While 20- and 22-carbon conjugated fatty acids have less efficacy on body fat reduction than CLA, a 19-carbon cognate of CLA, conjugated nonadecadienoic acid (CNA), has shown greater efficacy on body fat reduction compared to control in animal and tissue culture models [6,7].

Based on the suggested mechanism of CLA on body fat reduction, one can speculate the potential mechanism of CNA on the same. Previously, it was suggested that CLA reduced body fat, by reducing either fat accumulation or existing fat, and may involve multiple mechanisms: increasing energy expenditure, modulating adipocyte metabolism, and/or increasing fatty acid β-oxidation in skeletal muscle [1,8,9]. However, the mechanisms of CNA, particularly in comparison with CLA, have not been identified. Thus, the purpose of this report is, first, to directly compare the effectiveness of dietary CNA on body fat reduction compared to CLA and, second, to investigate the mechanisms of action for CNA to compare them with those of CLA.

## 2. Materials and methods

### 2.1. Materials

*cis*-10,*cis*-13 Nonadecadienoic acid was purchased from Nu-Chek Corp. (Elysian, MN) and CLA was provided by Natural Lipids Ltd. AS (Hovdebygda, Norway). The purity of CLA was 80.7% CLA (37.8% *cis*-9,*trans*-11, 37.6% *trans*-10,*cis*-12 and 5.3% other isomers), 13.7% oleic acid, 3.2% stearic acid, 0.4% palmitic acid and 0.2% linoleic acid. Semipurified diet (TD05350) was from Harlan Teklad (Madison, WI). Serum cholesterol kits [total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL)], triacylglycerides (TGs) and non-esterified fatty acids kits were purchased from Equal Diagnostics (Exton, PA). Tumor necrosis factor α (TNF-α), leptin and adiponectin kits were from R&D Biosciences (San Jose, CA). Other solvents used were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburg, PA).

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Table 1
PCR primers

| Gene name | Gene symbol | Reference sequences | Tissues |
|---|---|---|---|
| PPAR-γ | Pparg | NM_011146.2 | Adipose |
| PPAR-α | Ppara | NM_011144.5 | Hepatic |
| ACC | Acac | NM_133904.2 | Adipose, hepatic |
| FAS | Fas | NM_007988.3 | Adipose, hepatic |
| HSL | Lipe | NM_001039507.1 | Adipose |
| GLUT-4 | Slc2a4 | NM_009204.2 | Adipose, muscle |
| UCP-2 | Ucp2 | NM_011671.3 | Adipose, muscle |
| Leptin | Lep | NM_008493.3 | Adipose |
| CPT-I | Cpt1b | NM_009948.2 | Muscle |
| GAPDH | Gapdh | NM_008084.2 | Adipose, muscle, and liver |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

### 2.2. Preparation of CNA

CNA was prepared from *cis*-10,*cis*-13-nonadecadienoic acid by alkali isomerization as previously reported [6]. The products were stored under nitrogen at −20°C. Analysis of prepared CNA was performed using the previous gas chromatographic method for CLA [10]. Fatty acid methyl esters were prepared by reaction with 4% HCl in methanol for 20 min at 60°C and identified by gas chromatography/mass spectrometer. Hewlett-Packard 6890 series GC System with Agilent 7683 Series Injector and Agilent 5973 Network Mass Selective Detector were used. A Supelcowax-10 fused silica capillary column (100 m×0.25 mm i.d., 0.25-μm film thickness) was used and oven temperature was programmed from 50°C to 190°C, increased 20°C per minute, held for 50 min, increased 10°C per minute to 220°C and held for 50 min. The purity of CNA was 99.0% (43.6% *cis*-10,*trans*-12, 43.7% *trans*-11,*cis*-13, 6.2% *trans*-10,*trans*-12/*trans*-11,*trans*-13 and 5.5% other isomers).

### 2.3. Animals and diet

Fifty female 129Sv/J retired breeders (average 229±34 days old) were from breeding colonies maintained at the University of Massachusetts Amherst. Animals were housed in individual wire-bottomed cages in a windowless room with a 12-h light–dark cycle, under a protocol approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst. The semipurified powdered diet (TD05350) was used containing 20 w/w% fat to reflect the average American diet with 40% calories from fat. The diet was composed as follows (ingredient, g/kg): casein, "vitamin-free" test, 229; corn starch, 229; soybean oil, 200; maltodextrin, 132; sucrose, 100; cellulose, 50; mineral mix, AIN-93-MX (TD84046), 42; vitamin mix, AIN-93-VX (TD94047), 12; L-cystine, 3; choline bitartrate, 3; and TBHQ (antioxidant), 0.04. Diets were prepared at the beginning of the study and kept at −20°C until use. Diet and water were provided ad libitum throughout the experiment. Fresh diet was provided three times a week. After 1 week adaptation, animals were randomly divided into five groups and fed one of the treatment diets for 29 days: control, 0.5% CLA (80.7% total CLA), 0.01%, 0.05% or 0.1% CNA (99%). CLA or CNA was replaced at the expense of soy bean oil to achieve isocaloric diets. Body weight and food intake were monitored weekly. At Day 28, animals were subjected to a glucose tolerance test after 4 h fasting and sacrificed at Day 29. Thus, body weights and food intake data are presented for 28 days.

### 2.4. Measurement of energy expenditure and respiratory quotient

Energy expenditure was measured by an indirect calorimetric method, using a Metabolic Monitoring System (Qubit System, Kingston, ON, Canada). Five to six animals from each group were randomly selected at week −1 and assigned to Groups 1 to 7 (Mondays to Sundays). Three to four of these animals were placed into this system individually once a week (same day of the week) for 24 h for 5 weeks, from week −1 to 4, with free access to diet and water during their stay in this

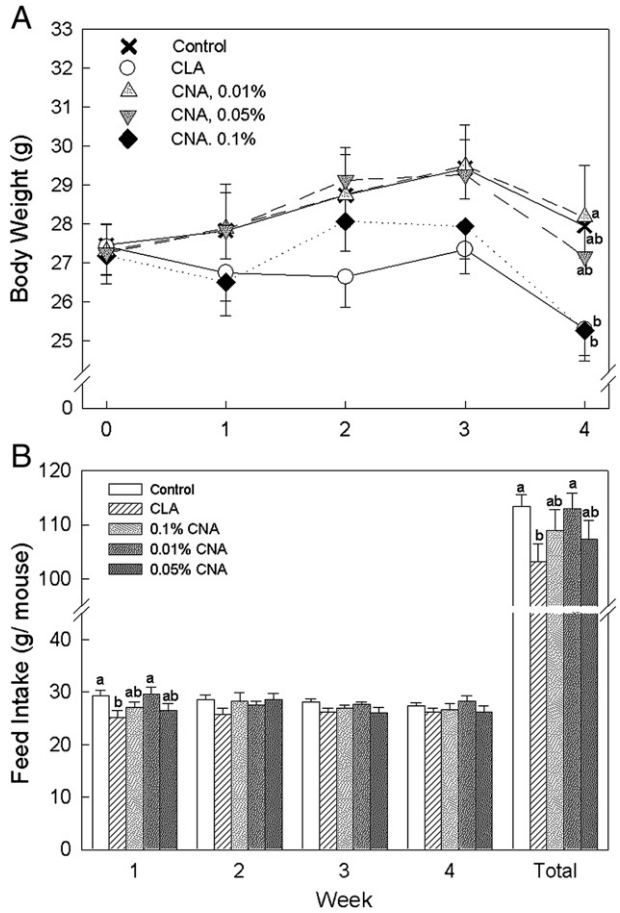



Fig. 1. Body weights (A) and food intake (B) after treatment with conjugated fatty acids. Female mice were fed one of the treatment diets for 29 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. For details, please see Materials and Methods section. Different letters at the same time point indicate significant difference at $P<.05$. Numbers are mean±S.E. ($n$=9–10).

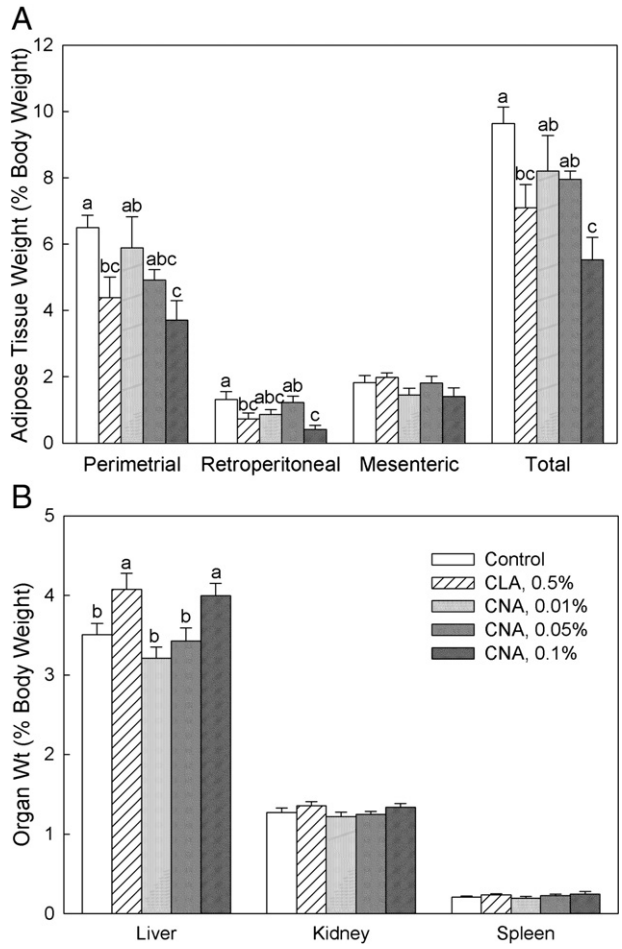



Fig. 2. Effects on organ weights after treatment with conjugated fatty acids. Adipose tissue weights (A) and liver, kidney and spleen weights (B) as % of body weights. Female mice were fed one of the treatment diets for 29 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. Different letters at the same organ weight indicate significant difference at $P<.05$. Numbers are mean±S.E. ($n$=9–10).

system. Data were presented as respiratory quotient (RQ) and energy expenditure (EE). RQ is the ratio of $VCO_2$ produced to $VO_2$ consumed. Energy expenditure at Week 4 was calculated from RQ data as: $EE=(3.815+1.232\times RQ)\times VO_2$ [11]. Energy expenditure was normalized to lean body weight and expressed as kilocalories per kilogram of lean body weight per hour.

### 2.5. Glucose tolerance tests

Glucose tolerance test was performed as described previously [12] on Day 28. Human glucose analyzer, Ascensia® CONTOUR® blood glucose meter, was used to measure glucose tolerance (Bayer HealthCare LLC, Mishakawa, IN). After 4-h fasting, glucose levels were monitored from tail vein, for Time 0, by using a glucose analyzer. Then, a sterile glucose solution was injected intraperitoneally (1g glucose/kg body weight as 300 g/L glucose solution). Next, blood glucose levels were measured from the tail vein by using a glucose analyzer at 15, 30, 60 and 90 min after administration of glucose. The areas under the curve (AUC) were calculated using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA).

### 2.6. Sacrifice and serum analyses

At the end of the feeding period, animals were sacrificed by $CO_2$ asphyxiation after 4 h fasting. Blood samples were collected by cardiac puncture, and the serum was separated by centrifugation at 1500×*g*. These serum samples were used for determination of cholesterol (total, HDL and LDL), TGs, non-esterified fatty acids, TNF-α, leptin and adiponectin using commercial kits as specified by the manufacturers. Internal organs (liver, kidney, spleen and adipose tissues) were also weighed.

### 2.7. Body composition analyses

At sacrifice, gut contents were removed and bodies minus two hindlegs, perimetrial fat and liver were freeze-dried and ground. Hindleg skeletal muscle (biceps femoris muscle from the one side), and half of the adipose (perimetrial) and liver tissue were weighed and frozen at −80°C for PCR analyses described below. The other halves of the adipose and liver tissues and one hindleg were freeze-dried, ground and combined with one half of ground body samples for body composition and stored in air-tight containers at −20°C. Total water contents were measuring by weight loss during freeze drying. Then total protein content was determined by the Kjeldahl method using a Kjeltec system (FOSS, Eden Prairie, MN) [13]. Total lipids were determined by the Soxhlet extraction method using diethyl ether with a Soxtec System (FOSS, Eden Prairie, MN). Ash was determined by gravitational method after incineration of dried sample with muffle furnaces at 550–600°C overnight.

### 2.8. mRNA expression analysis

From frozen tissues, mRNA expression levels of peroxisome proliferator-activated receptor α and γ (PPAR-α or PPAR-γ), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), hormone-sensitive lipase (HSL), glucose transporter 4 (GLUT-4), and uncoupling protein 2 (UCP2), leptin, and carnitine palmitoyltransferase I (CPT-I) were analyzed by real-time PCR using a Mx3000P QPCR System with MxPro Software (Stratagene, La Jolla, CA) and SYBR Green premix (SYBR GreenER, Invitrogen Corp., Carlsbad, CA). Total RNA from adipose tissue was extracted using Trizol reagent (Invitrogen Corp.) under RNase-free condition. Total RNA was reverse-transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (SuperScript II, Invitrogen Corp.). The reference sequence for the mRNAs, the housekeeping gene control (GAPDH) and respective tissues from where the expressions were determined are shown in Table 1. The primers were designed using Primer 3 Software (Version 0.4.0) based on the sequence deposited in the GeneBank. The nucleotide sequences of each primer and probe were Blast searched against the GeneBank database to confirm the uniqueness of each primer.

### 2.9. Statistical analysis

Data are shown as means and standard errors. All analyses were carried out using SAS software (Version 9.1.3, SAS institute Inc., Cary, NC) by the generalized linear model procedure and least square means options.

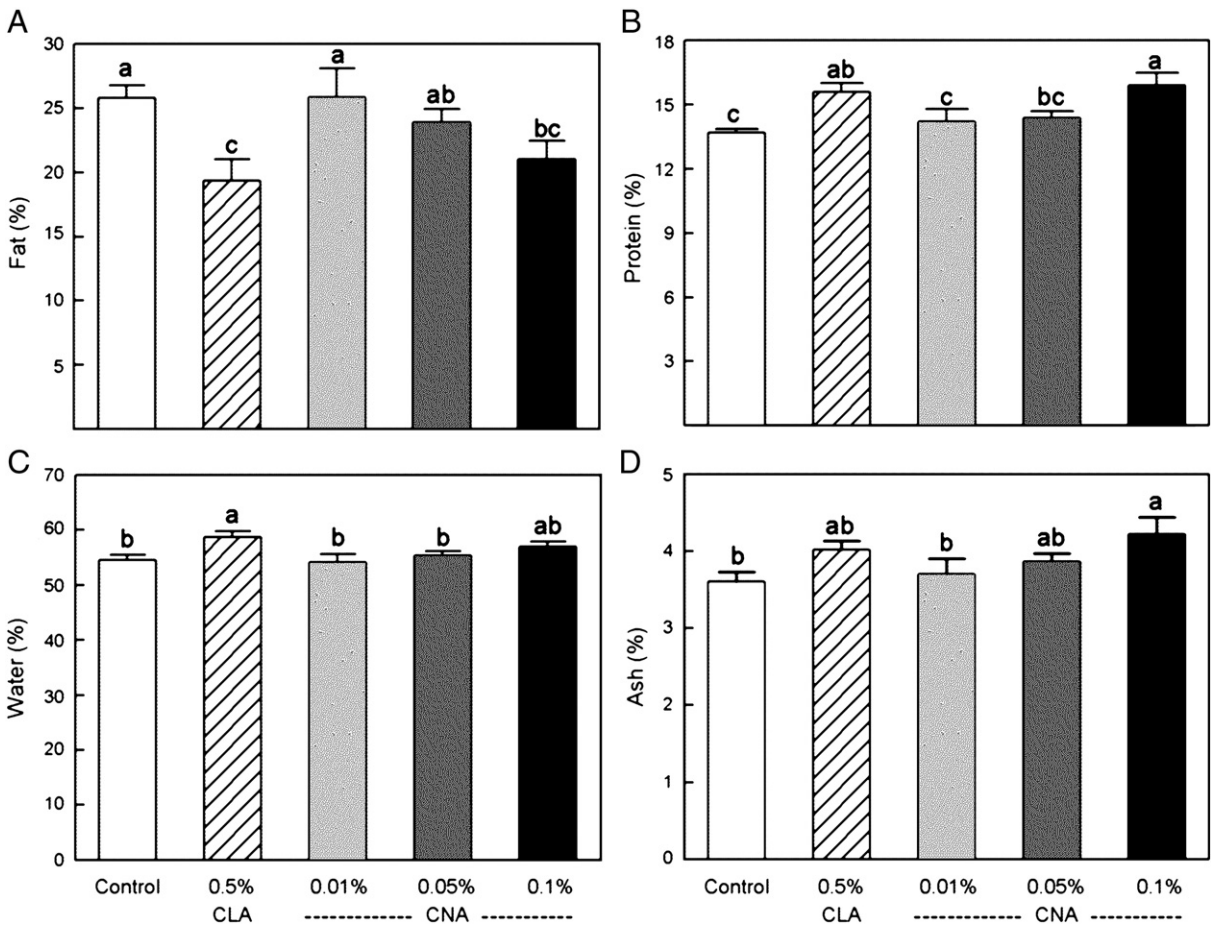



Fig. 3. Body composition modification by conjugated fatty acids. Total fat (A), protein (B), water (C) and ash (D). Female mice were fed one of the treatment diets for 29 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. Different letters in each figure indicate significant difference at $P<.05$. Numbers are mean±S.E. ($n=9–10$).

## 3. Results

### 3.1. Body weights and food intake

No significant difference in body weights was observed with the exception of Week 4, where 0.1% CNA- and CLA-fed animals had significantly lower weights compared to control (Fig. 1A). Reduced food intake was observed with CLA treatment compared to control at Week 1 and total but not with CNA treatments at all doses tested (Fig. 1B).

### 3.2. Tissue weights

As observed previously, CLA feeding significantly reduced adipose tissue weights (perimetrial, retroperitoneal, and total adipose tissues) compared to control. A similar trend was observed with dietary CNA treatment, with a significant reduction at the 0.1% level (Fig. 2A). The liver weights were significantly increased by dietary CLA as well as CNA (significant at 0.1% but not at 0.01% and 0.05%) (Fig. 2B). There were no differences in kidney and spleen weights between all treatments.

### 3.3. Body composition

Fig. 3A–D shows results of body composition represented as % of the empty carcass weights. CLA feeding, as previously reported, significantly reduced % body fat compared to control and effects of 0.1% CNA feeding on body fat was comparable to that of CLA (0.5%) (Fig. 3A). A dose-response of dietary CNA (0.01–0.1%) was observed with regard to body fat results (Fig. 3A). Dietary CLA and 0.1% CNA treatments enhanced % protein over control treatment (Fig. 3B). CLA feeding significantly increased % water while CNA feeding did not significantly influence % water at all doses tested (Fig. 3C). The 0.1% CNA supplement significantly increased % ash, while the CLA treatment group approached significance compared to control ($P$=.0745, Fig. 3D).

### 3.4. RQ and energy expenditure

RQ of the CNA (0.1%) group was significantly lower than control, from the second week of experiment, during both light and dark cycles (Fig. 4A–D). The lower doses of CNA treatment also significantly reduced RQ compared to control at Week 4, for both

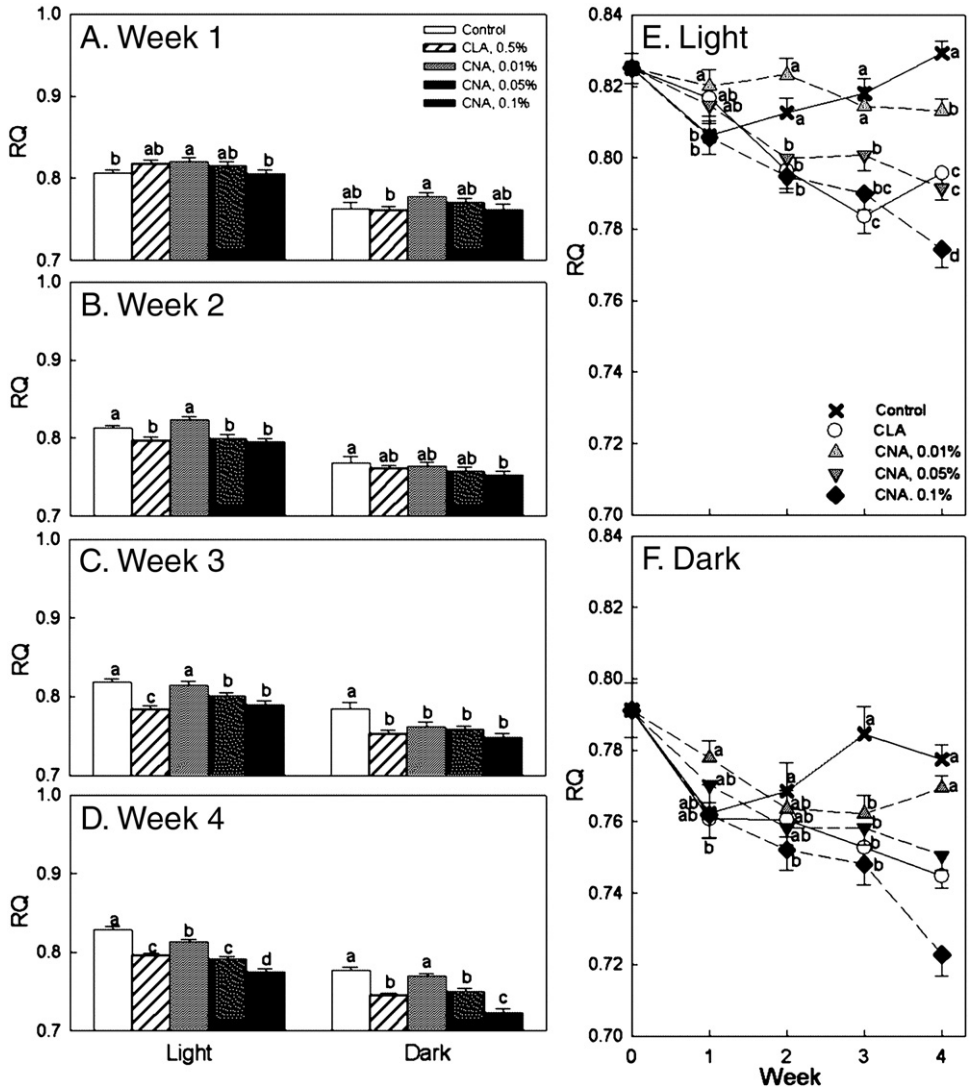



Fig. 4. RQ after treatment with CNA. Female mice were fed one of the treatment diets for 29 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. A through D represent RQ for each week after treatment during light and dark cycle. E and F are plots of these RQ over time periods. Different letters at the each variable and time point indicate significant difference at $P$<.05. Numbers are mean±S.E. ($n$=5–6).

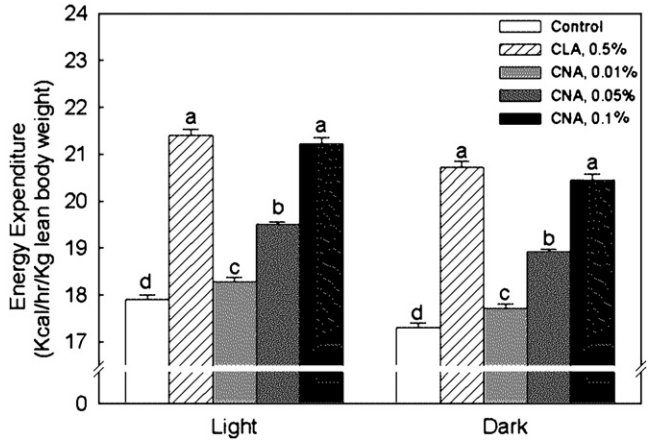



Fig. 5. Effects on energy expenditure by conjugated fatty acids. Female mice were fed one of the treatment diets for 4 weeks: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. Total energy expenditures were determined for 24 h during Week 4. Different letters at each variable indicate significant difference at $P<.0001$, except control vs. 0.01% CAN, which was $P<.05$ for light cycle and $P<.01$ for dark cycle. Numbers are mean±S.E. ($n=5$).

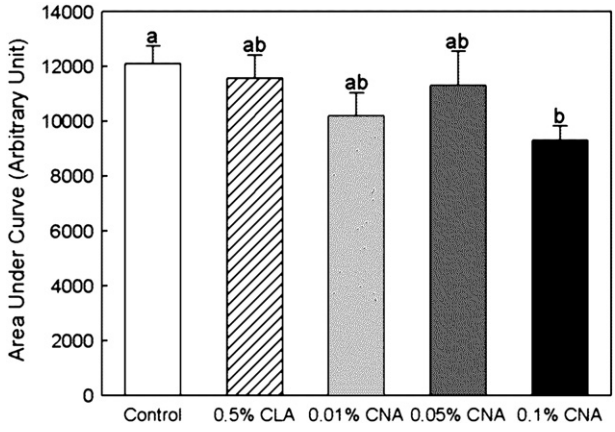



Fig. 6. Effects of conjugated fatty acids on glucose tolerance test as indicated by AUC. Female mice were fed one of the treatment diets for 28 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. Animals were fasted for 4 h and then injected with glucose, and the blood glucose levels were monitored at 0, 15, 30, 60 and 90 min. The AUCs were calculated and presented here. For details, please see Materials and Methods section. Different letters indicate significant difference at $P<.05$. Numbers are mean±S.E. ($n=9$).

0.01% and 0.05% CNA groups for light cycle and 0.05% CNA group for dark cycle (Fig. 4D). The CLA group showed a significant drop in RQ during light cycle at Week 2 and during dark cycle at Week 3, and this effect lasted until the end of the experiment (Fig. 4B). At Week 4, RQ of the 0.1% CNA group was significantly lower than the control and CLA groups (Fig. 4D). The overall trends over 4-week periods are shown in Fig. 4E for light cycle and Fig. 4F for dark cycle. This reduced trend of RQ over time indicates a shift of the main source of energy metabolism from carbohydrate to fatty acid, particularly significant for CNA (both 0.1% and 0.05%) and CLA treatments.

For total energy expenditure, both CNA at 0.1% and CLA treatments significantly increased total energy expenditure for both light and dark cycles; 18% and 20% increase over control, respectively (Fig. 5). Dietary inclusion of 0.1% CNA had an equivalent effect to 0.5% CLA, while lower doses of CNA, at 0.01% and 0.05%, showed dose responses and significant increase in energy expenditure; 2.2% ($P<.05$) and 9% ($P<.001$) above control, respectively.

### 3.5. Glucose tolerance tests

Glucose response during GTT was significantly improved by CNA treatment at the 0.1% level (Fig. 6). AUC was reduced 23% in the CNA treatment group (0.1%) compared to control, while CLA treatment was not different than control. Two other doses of CNA (0.01% and 0.05%) had no effect on AUC.

### 3.6. Blood parameters

Results from various blood parameters are shown in Table 2. Feeding CNA or CLA did not affect levels of TG, total cholesterol, HDL-cholesterol, LDL-cholesterol, the ratio of HDL/LDL or glucose (Table 2). Non-esterified fatty acid (NEFA) levels were significantly reduced by dietary CNA (at 0.05% and 0.1%), while there was no difference in NEFA with CLA or 0.01% CNA treatments compared to control. Reduction of TNF-α was the greatest with CLA feeding, while 0.1% and 0.05% CNA treatments also significantly reduced this compared to control but less than that of the CLA group. No differences were observed in levels of adiponectin with either CNA or CLA compared to control, while leptin was significantly reduced by CNA (0.05% and 0.1%) or CLA treatments.

### 3.7. Effects on lipid metabolism

To elucidate the effects of dietary CNA or CLA on glucose and lipid metabolism, mRNA levels of key genes involved in these processes were determined. As key adipogenesis genes, ACC, FAS and PPAR-γ were determined from white adipose tissues (Fig. 7A–C). CLA significantly increased expression of ACC and FAS in adipose tissues, while CNA dose-dependently increased expression of these genes, although they were significantly lower than those of CLA-treated group. Meanwhile, both CLA and 0.1% CNA treatments significantly reduced expression of PPAR-γ in white adipose tissue compared to control (Fig. 7C). CNA at all doses tested, but not CLA treatment, significantly increased expression of hormone sensitive lipase (the key enzyme for lipolysis) compared to control (Fig. 7D). As expected from serum leptin results, expression of leptin from white adipose tissue was significantly reduced by CLA and CNA (at all levels tested) treatments (Fig. 7E). Expression of UCP2, the key marker for energy expenditure in white adipose tissue and muscle, was increased by CLA and 0.1% CNA treatments (Figs. 7F and 8B). Both CLA and 0.1% CNA treatments significantly increased expression of CPT-I in muscle, which is the key for fatty acid β-oxidation (Fig. 8A). CLA and 0.1% CNA

Table 2
Effects of conjugated fatty acids on serum parameters[1]

| | Control | 0.5% CLA | 0.01% CNA | 0.05% CNA | 0.1% CNA |
|---|---|---|---|---|---|
| TG (mg/dl) | 160±12 | 174±15 | 261±59 | 148±11 | 177±22 |
| Cholesterol (mg/dl) | 172±20 | 233±11 | 152±29 | 188±15 | 176±15 |
| HDL-cholesterol (mg/dl) | 85±8 | 85±12 | 87±9 | 69±9 | 71±9 |
| LDL-cholesterol (mg/dl) | 106±1 | 99±4 | 101±2 | 100±2 | 91±8 |
| HDL to LDL | 0.81±0.08 | 0.88±0.12 | 0.87±0.10 | 0.75±0.10 | 0.82±0.09 |
| Glucose (mg/dl) | 61.2±5.9 | 59.5±4.8 | 50.0±3.7 | 56.0±6.9 | 50.2±5.3 |
| NEFA (mmol/L) | $3.88\pm0.57^{a}$ | $3.16\pm0.24^{ab}$ | $2.98\pm0.49^{b}$ | $1.87\pm0.29^{b}$ | $1.88\pm0.18^{b}$ |
| TNF-α (ng/L) | $286\pm17^{ab}$ | $157\pm4^{d}$ | $297\pm8^{a}$ | $266\pm8^{b}$ | $194\pm5^{c}$ |
| Leptin (μg/L) | $4.11\pm0.60^{a}$ | $1.42\pm0.28^{b}$ | $2.63\pm1.24^{ab}$ | $1.32\pm0.29^{b}$ | $0.54\pm0.15^{b}$ |
| Adiponectin (mg/L) | 3.69±0.09 | 3.60±0.09 | 3.92±0.09 | 3.68±0.15 | 3.56±0.09 |

[1]Female mice were fed one of the treatment diets for 29 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. Animals were fasted for 4 h prior to sample collection. Different letters in the same row indicate significant difference at $P<.05$. Numbers are mean±S.E. ($n=4$–9).

treatments significantly increased expression of PPAR-α in the liver (Fig. 9A). Hepatic ACC expressions was increased by CNA (with the greatest effect at the 0.1% level) and CLA treatments, while hepatic FAS expression in CNA and CLA groups were significantly lower than control (Fig. 9B and C).

As a marker for glucose metabolism, we also measured GLUT-4 in white adipose tissues, and both CLA and 0.1% CNA treatment significantly reduced its expression (Fig. 7G). CLA and 0.05% and 0.1% CNA treatments also significantly increased expression of GLUT-4 in skeletal muscle (Fig. 8C). CNA showed dose-dependent effects on GLUT-4 expression in both adipose tissue and muscle (Figs. 7G and 8C).

## 4. Discussion

It has been previously reported that the effect of CNA on body fat regulation is more efficient than that of CLA [6]. In this report, we confirm that observation and additionally determined that dietary CNA is five times more potent than CLA. In addition, results in this report indicate that CNA has a similar mechanism of action as CLA on lipid metabolism and fat reduction. The major difference between CNA and CLA is on lipolysis, as indicated by increased HSL expression in white adipose tissue by CNA, but not CLA.

It has been suggested that the reduced body fat or weight gain due to CLA may involve multiple mechanisms [1,8,9]. First, numerous studies have shown that CLA increases energy expenditure as shown by increased oxygen consumption, increased heat loss and/or increased expression of uncoupling proteins, which are markers for energy expenditure[14-18]. Our data clearly indicate that both CNA and CLA treatments significantly increased total energy expenditure and also increased UCP-2 expression in white adipose tissue and muscle, thus supporting the above mentioned observation (Figs. 7F and 8B). Even lower levels of CNA supplementation (0.01% and 0.05%) significantly increased energy expenditure; thus with prolonged exposure, these doses of CNA may also result in significant reduction of body fat.

It has been also suggested that CLA increases fatty acid β-oxidation by enhancing activity and expression of CPT-I, which is the rate-limiting enzyme for this process, particularly in skeletal muscle [1,19,20]. In our results here, both CNA and CLA significantly increased expression of CPT-I in skeletal muscle (Fig. 8A), which suggests that both CNA and CLA promote fat consumption as an energy source rather than storage. This is also supported by the fact that both CNA and CLA decreased the RQ of animals (Fig. 4), reflecting the increased lipid, rather than carbohydrate, consumption as an energy source. This observation is consistent with West et al. [21] and may be a part of the mechanisms that would also contribute to CNA's and CLA's effects on fat mass reduction.

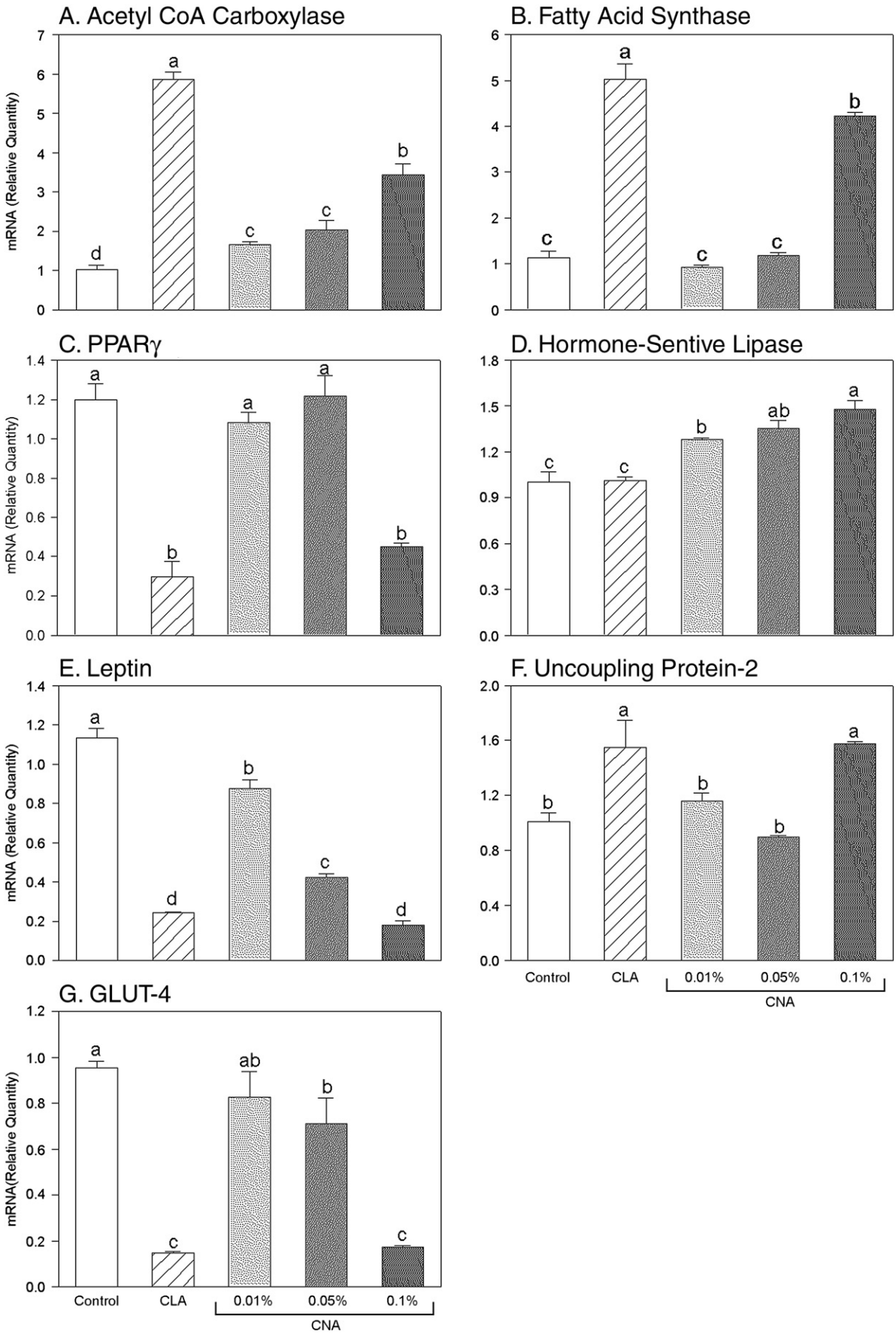
Additionally, CLA is known to modulate fat storage. For example, both CNA and CLA have been reported to reduce fat uptake by inhibiting lipoprotein lipase activity in 3T3-L1 adipocytes, where CNA had similar inhibitory effect on lipoprotein lipase activity compared to CLA [6,10,19]. Effects of CLA on HSL have not been consistent; expression increased in 3T3-L1 adipocytes, decreased in human adipose tissue, or not changed in hamster adipose tissue [22-24]. In this report, CNA, but not CLA treatment as low as 0.01% of diet, showed significant increased HSL expression in white adipose tissue, reflecting increased lipolysis (Fig. 7D). This may help explain in part the greater efficacy of CNA on body fat reduction compared to CLA. However, it needs to be pointed out that the reduction of body fat can be achieved through decreased fat accumulation and/or increased existing fat loss. Our results here do not clearly distinguish if CLA or CNA decreases fat accumulation or enhances fat loss over time.

Along with previous reports that CLA reduces lipogenesis in part by controlling nuclear factor-κB and PPAR-γ-mediated mechanisms [25-27], results in this report suggest that CNA may also influence adipocyte differentiation and apoptosis in manners similar to that of CLA (Fig. 7C). Although we have not monitored adipocyte apoptosis, as others have reported that CLA enhanced apoptosis of preadipocytes, we speculate that CNA may increase adipocyte apoptosis as well [1,28-30].

CLA is also known to modulate adipokines and/or cytokines. The adipokines and cytokines, such as leptin, TNF-α, and adiponectin are important hormonal regulators secreted by adipocytes. They are known to be involved in controlling food intake (leptin), obesity and glucose tolerance (TNF-α), and many chronic pathologies including insulin sensitivity (adiponectin and/or leptin) [31,32]. Previously, CLA reduced expression and/or secretion of leptin and TNF-α in serum, adipose tissue and/or the liver, although effects of CLA on adiponectin were not consistent [33-37]. In this report, we observed that effects of CNA on these parameters were similar to CLA; reduced leptin and TNF-α but not adiponectin. Compared to CLA, CNA has greater potency on significantly reducing leptin at 0.05% of diet compared to control (Table 2). Thus, modulation of these cytokines by CNA may also impact its biological significance, similar to CLA. With these mechanisms, CNA as well as CLA effectively reduced total body fat in this animal model.

Compared to the body fat reduction seen in mice, there are limited responses to CLA in other species; rats (particularly males), pigs and especially humans [1,9,16]. It has been estimated that mice fed diet containing 0.5 w/w% CLA is equivalent to about 56 g CLA/day per 70 kg for humans, where typically about 3–4 g per day (the range was between 0.7 and 6.8 g/day) was used for human studies [1,38]. Thus, differences in CLA's effectiveness on fat reduction in different models may be due in part to the relatively low dose used in human studies compared to that used in mice. In fact, Whigham et al. [39] previously showed a positive correlation with dose of CLA supplement and efficacy on body fat reduction based on a meta-analysis. This implies that for a compound with greater potency than CLA, such as CNA, about 3 g per day or less may have the potential to result in greater efficacy with regard to body fat reduction. However, there are additional explanations of the differences in CLA's effects on fat control, such as differences in metabolism (higher metabolic rate in mice than other species), differences in experimental design (reducing fat accumulation vs. preexisting fat) and differences in dietary regimes (ad libitum vs. calorie restriction) [40-43]. Thus, further investigations are needed to confirm that CNA is effective in different models.

It is important to point it out here that both the CLA and CNA used in this study are mixtures of isomers; the main isomers were the *cis*-9, *trans*-11 and *trans*-10,*cis*-12 for CLA and *cis*-10,*trans*-12 and *trans*-11, *cis*-13 for CNA. Based on the observation that the *trans*-10,*cis*-12 CLA isomer is responsible for body fat reduction in mice [1], one can speculate that the active isomer for CNA would be the comparable, *trans*-11,*cis*-13 isomer. However, the structure–activity relation study indicated that *trans*-10 and *cis*-12 conjugation is the key structure for this activity [3]; thus, it is possible that the isomer with the same double bond location, the *cis*-10,*trans*-12 CNA, may have significance with regard to lipid metabolism. Previously, it was reported that conjugated eicosadienoic acid ($\Delta 20{:}3^{c11,t13/t12,c14}$) showed activities similar to those of CLA; however, it was determined that CLA converted from this conjugated fatty acid in the biological system was the origin of original activities [7]. However, it will be unlikely that CNA will be converted to CLA or vice versa due to the one-carbon difference between these two conjugated fatty acids. In fact, we have previously analyzed mouse tissues fed CNA (0.3% dietary level) for 2 weeks and found that CNA was indeed incorporated in animal tissue, while there was no detectable CLA nor potential fatty acid β-oxidative metabolites of CNA (such as conjugated 17-carbon fatty acids). However, this does not eliminate the possibility that they were under the detection limit due to the fact that CNA levels were


A. Acetyl CoA Carboxylase
B. Fatty Acid Synthase
C. PPARγ
D. Hormone-Sentive Lipase
E. Leptin
F. Uncoupling Protein-2
G. GLUT-4
mRNA (Relative Quantity)
mRNA(Relative Quantity)
Control
CLA
0.01%
0.05%
0.1%
CNA

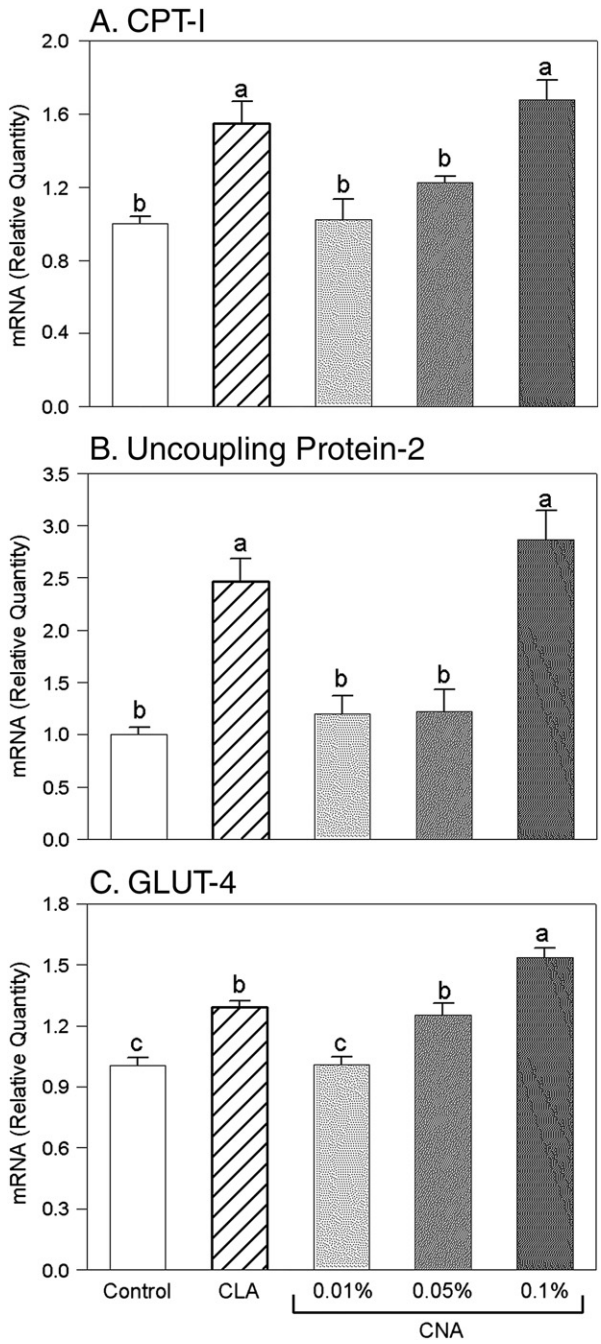



Fig. 8. Effects of conjugated fatty acids on expression of selected genes from the skeletal muscle: (A) CPT-I, (B) UCP2 and (C) GLUT-4. Female mice were fed one of the treatment diets for 29 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. Different letters in each figure indicate significant difference at $P<.05$. Numbers are mean±S.E. ($n=6$).

low in those samples (unpublished data). Thus, we speculate that there are common recognition sites or targets for CNA, CLA, or their metabolites, which can drive these biological activities. Further studies with pure CNA isomers in comparison to CLA isomers are needed to determine the key isomer or active metabolites for CNA.

It has been reported that the effects of CLA on bone mass are inconsistent, and with additional calcium would improve CLA's effect on bone mass [44]. In this study, we again observed that CLA treatment slightly increased total ash (as representative of bone mass), which approaches significance (Fig. 3D, $P=.0745$ compared to control). However, 0.1% CNA treatment significantly increased total ash. This may suggest that CNA has greater potency than CLA for improving bone mass; however, more studies are needed to confirm this, along with studies of calcium interaction with CNA.

Previously, CLA has been linked to reduced serum cholesterol levels; however, we did not observe any effects of CLA or CNA on cholesterol or TG in this study. Inconsistent observations regarding CLA's effects on cholesterol may be due to differences in study designs (mice vs. rabbits or hamsters; normal vs. hypercholesterolemic animals; high cholesterol diet vs. normal; age of animals) [9,45-47]. It is not clear at this moment if CNA has similar effects on cardiovascular disease risk as CLA.

The main concerns over CLA use identified are fatty liver and glucose intolerance [2,48,49]. As observed here as increased liver weights, feeding both CNA at 0.1% and CLA resulted in enlarged liver. Since effects of CNA and CLA in our results were similar, it is possible that the effects of CNA on the liver are caused by mechanisms similar to CLA as observed by increased hepatic PPAR-α expression by 0.1% CNA and CLA treatments, resulting in increased hepatic lipogenesis. In addition, increased liver weight was only observed with treatments with significant reduction of body fat, also the 0.1% CNA and CLA treatments. Thus, the reduction of lipid deposition in extrahepatic tissues may also have contributed to the enlarged liver. These findings are consistent with observations in previous reports [30,35,50,51]. However, others reported no effects by CLA on the liver or reduced hepatic steatosis in a rat model [52,53], and O'Hagan & Menzel [54] suggested increased liver weight by CLA may be a temporary response of biological systems to CLA and potentially reversible. Thus, further studies with long-term feeding are needed to confirm if the enlarged liver would be different from short-term studies. In addition, since CNA treatments lower than 0.1% showed a tendency of reduced body fat in 4 weeks with slight but significant increase of energy expenditure in our experiment, long-term effects of low doses of CNA (less than 0.05%) on body fat and enlarged liver needed to be determined.

The other main concern over the use of CLA is on its effect on glucose homeostasis. Effects of CLA on glucose homeostasis have not been consistent. In contrast to impaired insulin sensitivity in normal or nondiabetic animal or human models [1,30,55], CLA has been shown to improve glucose metabolism as shown by decreasing glucose or insulin concentrations, or glucose tolerance in diabetic or obese models [1,12,18,56-58]. The mechanism of insulin resistance by CLA may be due in part to enhanced fatty acid β-oxidation [59]. In the current report, with relatively old mice, we did not see any difference in glucose levels or glucose tolerance tests by CLA, while 0.1% CNA improved glucose tolerance compared to control. In addition, expression of GLUT-4 in the muscle was increased by CNA and CLA, where 0.1% CNA was more effective than CLA (Fig. 8C). Although CLA's effect on insulin resistance may be temporary as reported [12,54], the difference between CNA and CLA in glucose tolerance tests suggests that CNA may be more beneficial in glucose homeostasis than CLA. Decreased NEFA levels by CNA may casually contribute to its effects on glucose metabolism, but not CLA. It has been reported that increased serum NEFA stimulates hepatic gluconeogenesis, thus increasing glucose concentration [60]. Thus, decreased NEFA by CNA, but not CLA, may help explain decreased GTT response by CNA.

In conclusion, our results indicate that dietary CNA effectively reduces body fat compared to CLA in this model with mechanisms

Fig. 7. Effects of conjugated fatty acids on expression of selected genes from the white adipose tissue: (A) ACC, (B) FAS, (C) PPAR-γ, (D) HSL, (E) leptin, (F) UCP2 and (G) GLUT-4. Female mice were fed one of the treatment diets for 29 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. Different letters in each figure indicate significant difference at $P<.05$. Numbers are mean±S.E. ($n=6$).

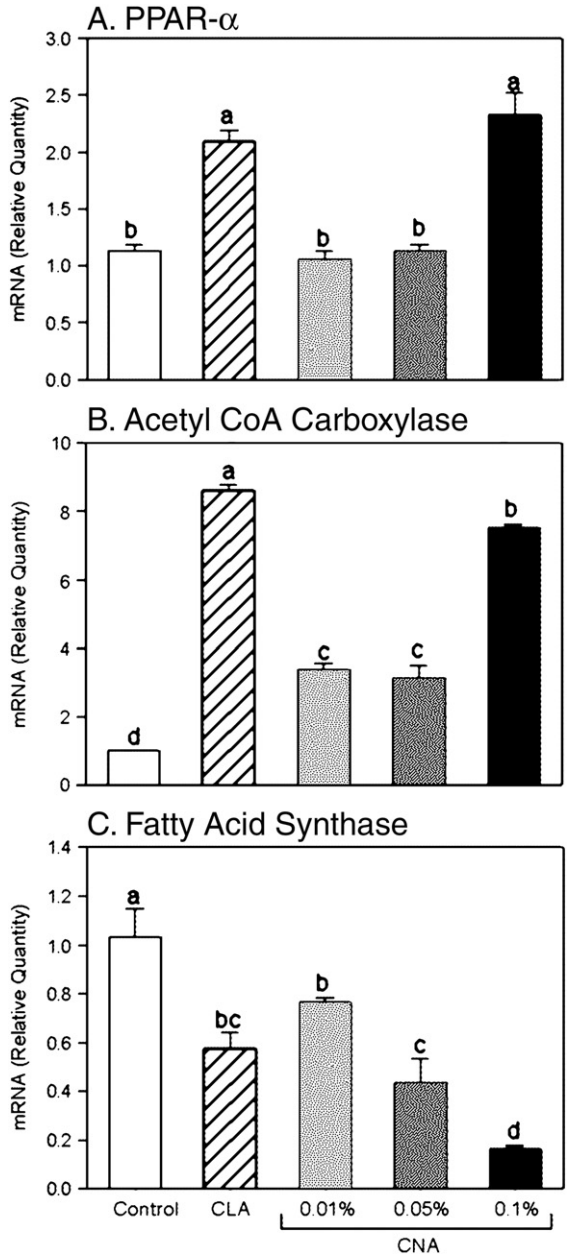



Fig. 9. Effects of conjugated fatty acids on expression of selected genes from the liver: (A) PPAR-α, (B) ACC, and (C) FAS. Female mice were fed one of the treatment diets for 29 days: control, 0.5% CLA, or 0.01%, 0.05%, and 0.1% CNA. Different letters in each figure indicate significant difference at $P<.05$. Numbers are mean±S.E. ($n=6$).

similar to those of CLA. Additionally, lower doses of CNA, as low as 0.01% of diet, significantly increase energy expenditure without adverse effects on the liver and glucose metabolism. Further studies with different conditions as well as determination of isomer effects are needed to evaluate the potential future application of CNA. Currently, CNA has not been tested in human; however, results from this study suggest that CNA has potential to be used as a pharmacological agent to assist current efforts to reduce the obesity epidemic.

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