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ORIGINAL ARTICLE

Dietary t10,c12-CLA but not c9,t11 CLA Reduces Adipocyte Size in the Absence of Changes in the Adipose Renin–Angiotensin System in *fa/fa* Zucker Rats

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Abstract In obesity, increased activity of the local renin–angiotensin system (RAS) and enlarged adipocytes with altered adipokine production are linked to the development of obesity-related health problems and cardiovascular disease. Mixtures of conjugated linoleic acid (CLA) isomers have been shown to reduce adipocyte size and alter the production of adipokines. The objective of this study was to investigate the effects of feeding individual CLA isomers on adipocyte size and adipokines associated with the local adipose RAS. Male *fa/fa* Zucker rats received either (a) control, (b) *cis*(c)9,*trans*(t)11-CLA, or (c) t10,c12-CLA diet for 8 weeks. The t10,c12-CLA isomer reduced adipocyte size and increased cell number in epididymal adipose tissue. RT-PCR and Western blot analysis revealed that neither CLA isomer altered mRNA or protein levels of angiotensinogen or AngII receptors in adipose tissue. Likewise, levels of the pro-inflammatory cytokines TNF-α and IL-6 or the anti-inflammatory cytokine IL-10 were unchanged in adipose tissue. Similarly, neither CLA isomer had any effect on phosphorylation nor DNA binding of NF-κB. Our results suggest that although the t10,c12-CLA isomer had beneficial effects on reducing adipocyte size in obese rats, this did not translate into changes in the local adipose RAS or associated adipokines.

Keywords Angiotensinogen · Adipokines · Adipocyte · Conjugated linoleic acid · Adipose · Obesity

Abbreviations

AngII	Angiotensin II
ANOVA	Analysis of variance
c	*cis*
CLA	Conjugated linoleic acid
IκBα	Inhibitor protein kappa-B alpha
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor kappa-B
RAS	Renin–angiotensin system
rp	Ribosomal protein
t	*trans*
TNF	Tumor necrosis factor

Introduction

It is well known now that adipose tissue not only stores triglycerides for energy but also functions as an endocrine organ participating in energy homeostasis, neuroendocrine control and immune functions [1, 2]. Increases in adipose tissue, such as those observed in obesity, are associated

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with enlargement (hypertrophy) of adipocytes, macrophage accumulation and subsequent changes in the production of bioactive proteins or adipokines to favor a more pro-inflammatory profile [3–5]. These changes are associated with obesity-related health problems, the development of metabolic syndrome and an increased risk of cardiovascular disease [6–8]. Some of the pro-inflammatory adipokines involved in pathogenesis of these diseases include angiotensinogen, tumor necrosis factor (TNF)-α and interleukin (IL)-6.

All components of the renin–angiotensin system (RAS), including angiotensin II (AngII) and its precursor angiotensinogen, are present in adipose tissue and this local RAS has been implicated in the regulation of adipose growth as well as the regulation of blood pressure [9, 10]. In support of this argument, mice that selectively over-express angiotensinogen in adipose tissue exhibit an increase in circulating angiotensinogen and are hypertensive [9]. Upon binding to its receptor, AngII also stimulates pro-inflammatory cytokines through the activation of nuclear factor-κB (NF-κB) [11], which further contributes to the pathogenesis of cardiovascular complications [12].

Due to the large increases in obesity-related cardiovascular complications, the need for preventative strategies is at an all time high. The use of dietary compounds to prevent or treat obesity-related cardiovascular risk factors may be an attractive idea for some. Conjugated linoleic acid (CLA) is a fatty acid derived from ruminant animals that is being examined for its potential health benefits [13]. Our laboratory has shown that feeding a mixture of CLA isomers to obese *fa/fa* Zucker rats favorably alters levels of adipokines in serum and epididymal adipose tissue, and at the same time reduces adipocyte size [14]. A recent study by Martins et al. [15] also showed increased serum adiponectin levels in obese Zucker rats given a mixture of CLA isomers and the potential for other cardiovascular benefits such as lowered plasminogen activated inhibitor-1 levels. At this time, however, it remains unclear which CLA isomer is responsible for reducing adipocyte size in an obese model and if other adipose-derived proteins may be affected. Research from other groups suggest that the t10,c12-CLA isomer may be responsible for the cardiovascular benefits, such as improving blood pressure by reducing adipose angiotensinogen mRNA [16]. At this point, however, it remains unclear whether these changes in angiotensinogen mRNA will affect angiotensinogen protein levels or if CLA would have an effect on AngII receptors. Additionally, previous studies examining CLA and NF-κB have primarily been carried out in culture models [17, 18], leaving the effects of CLA isomers in vivo on NF-κB activation in tissues still to be determined. This study was therefore designed to investigate the effects of individual CLA isomers on epididymal adipocyte size and to examine the relationship with the local adipose RAS in obesity. The study focused on epididymal adipose tissue as a visceral adipose depot because visceral adiposity is associated with metabolic complications of obesity and cardiovascular disease and is strong predictor of obesity-related risk factors and mortality [19, 20].

Materials and Methods

Animals and Diet

During an 8-week experimental period, 6-week old male *fa/fa* Zucker rats ($n = 6$/group; Harlan, Indianapolis, IN, USA) received diets based on the AIN-93 formula differing in the amounts of CLA isomers as previously published [21]. There were three groups (a) control diet (b) 0.4% (w/w) *cis*(c)9,*trans*(t)11-CLA diet, and (c) 0.4% (w/w) t10,c12-CLA diet. At the end of the feeding period, rats were euthanized by CO_2 asphyxiation and epididymal adipose tissue was dissected, frozen in liquid nitrogen or Cryogel embedding medium and subsequently stored at −80 °C until analyzed. The University of Manitoba Protocol Management and Review Committee approved the animal protocol which was in agreement with the Canadian Council on Animal Care Guidelines [22].

Morphometry

Tissues sections (10 μm thick) were cut on a cryotome and mounted on SuperFrost Plus glass slides. Sections were fixed in formaldehyde and assessed under a light microscope (BH2-RFCA; Olympus) equipped with a camera (Q-Imaging). Using Image J software as previously described [14], a continuous block of 25 cells was measured in every field to determine average cell size (μm^2) and mean adipocyte size per group was calculated. Based on Sturges' rule [23], 10 different classes of adipocyte area were determined and the cell size distribution for each treatment group was expressed as a percentage. The number of adipocyte was assessed by counting a 160 μm^2 area in the centre of each field/section and the mean number per group was calculated.

RT-PCR Analysis

Total RNA was isolated from epididymal adipose tissue using TRIzol reagent. The concentration of RNA and the purity of the samples were assessed spectrophotometrically. RNA was digested with DNase, reverse transcribed into cDNA and amplified with an Access RT-PCR system kit (Promega). Primer sequences for detection of angiotensinogen, AngII receptor1a, AngII receptor1b, TNF-α, IL-6,

Table 1 Primer sequences for RT-PCR

Gene name	Primer sequence
Angiotensinogen	F: 5′-CAC GGA CAG CAC CCT ATT TT-3′
	R: 5′-GCT GTT GTC CAC CCA GAA CT-3′
TNF-α	F: 5′-GTC AGC CGA TTT TGC CAT TTC-3′
	R: 5′-AAC GAT GAA CAC GCC AGT-3′
IL-6	F: 5′-CCC AAC TTC CAA TGC TCT CCT AAT G-3′
	R: 5′-GCA CAC TAG GTT TGC CGA GTA GAC C-3′
IL-10	F: 5′-GGC TCA GCA CTG CTA TGT TGC C-3′
	R: 5′-AGC ATG TGG GTC TGG CTG ACT G-3′
rpL32	F: 5′-TAA GCG AAA CTG GCG GAA AC-3′
	R: 5′-GCT CGT CTT TCT ACG ATG GCT T-3′
AngII receptor type 1a	F: 5′-CTC AAG CCT GTC TAC GAA AAT GAG-3′
	R: 5′-TAG ATC CTG AGG CAG GGT GGA T-3′
AngII receptor type 1b	F: 5′-CTT TCC TAC CGC CCT TCA GAT A-3′
	R: 5′-TGA GTG CTT TCT CTG CTT CAA C-3′

F Forward primer, *R* reverse primer

IL-10 and ribosomal protein L32 (rpL32) mRNA can be found in Table 1. In view of the fact that high levels of pro-inflammatory mediators have been linked to obesity and CLA is considered effective against obesity [24], we also examined the anti-inflammatory mediator IL-10. cDNA products were run on an agarose gel, visualized with Vista Green Nucleic Acid Stain and relative intensity of the bands was quantified by densitometry. Results are expressed as arbitrary units after normalization to rpL32 expression.

Protein Extraction and Western Blotting

The bicinchoninic acid assay (Pierce) was used to determine total protein in samples isolated from adipose tissue using a mortar and pestle and 3× sodium dodecyl sulfate sample buffer. Adipose samples (15 μg protein) were analyzed by Western blotting as described previously [25] with antibodies (diluted 1:1,000) for angiotensinogen (Fitzgerald), AngII receptor 2 (Santa Cruz), TNF-α (Cell Signaling), IL-6 (Biosource), IL-10 (Biosource), NF-κB (Cell Signaling), F4/80 (Abcam) and total mitogen-activated protein kinase (MAPK) (Cell Signaling). The latter was used to account for possible variability in sample loading. Autoradiography and scanning densitometry with Quantity One image analysis software (BioRad) were used to capture and quantify band intensities.

NF-κB Activation

Nuclear extracts from epididymal adipose tissue samples were prepared using a Nuclear Extract Kit according to manufacturer's instructions (Active Motif). NF-κB binding activity was determined using a TransAM NF-κB Transcription Factor ELISA Kit according to manufacturer's instructions (Active Motif).

Statistical Analysis

Data were analyzed with Statistical Analysis Software (SAS 6.04; SAS Institute, Cary, NC, USA) to examine the effect of diet on cell size, mRNA and protein levels by a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significant at $P < 0.05$ and all results are expressed as means ± standard error (SEM).

Results

Growth Performance and Feed Intake

There were no significant differences in body weight gained (control 433 ± 10 g; c9,t11-CLA 452 ± 9 g; t10,c12-CLA 453 ± 8 g) or epididymal adipose mass (data not shown) among groups. Likewise, daily food intake was similar among groups (control 27.2 ± 0.5 g/day; c9,t11-CLA 28.8 ± 0.6 g/day; t10,c12-CLA 28.9 ± 0.4 g/day).

Adipocyte Size

Previously we reported that a mixture of CLA isomers reduced adipocyte size [14], however, the contribution of the individual CLA isomers was not investigated. Therefore, the current study was designed to examine the effect of individual CLA isomers on adipocyte size. The group receiving the t10,c12-CLA isomer had 60% smaller adipocytes compared to the control and c9,t11-CLA groups (Fig. 1a, b). The distribution pattern of cell size shows that 80% of adipocytes in the t10,c12-CLA isomer group were <2,000 μm^2, with less than 2% of cells >5,000 μm^2, whereas the control group and the c9,t11-CLA group had a

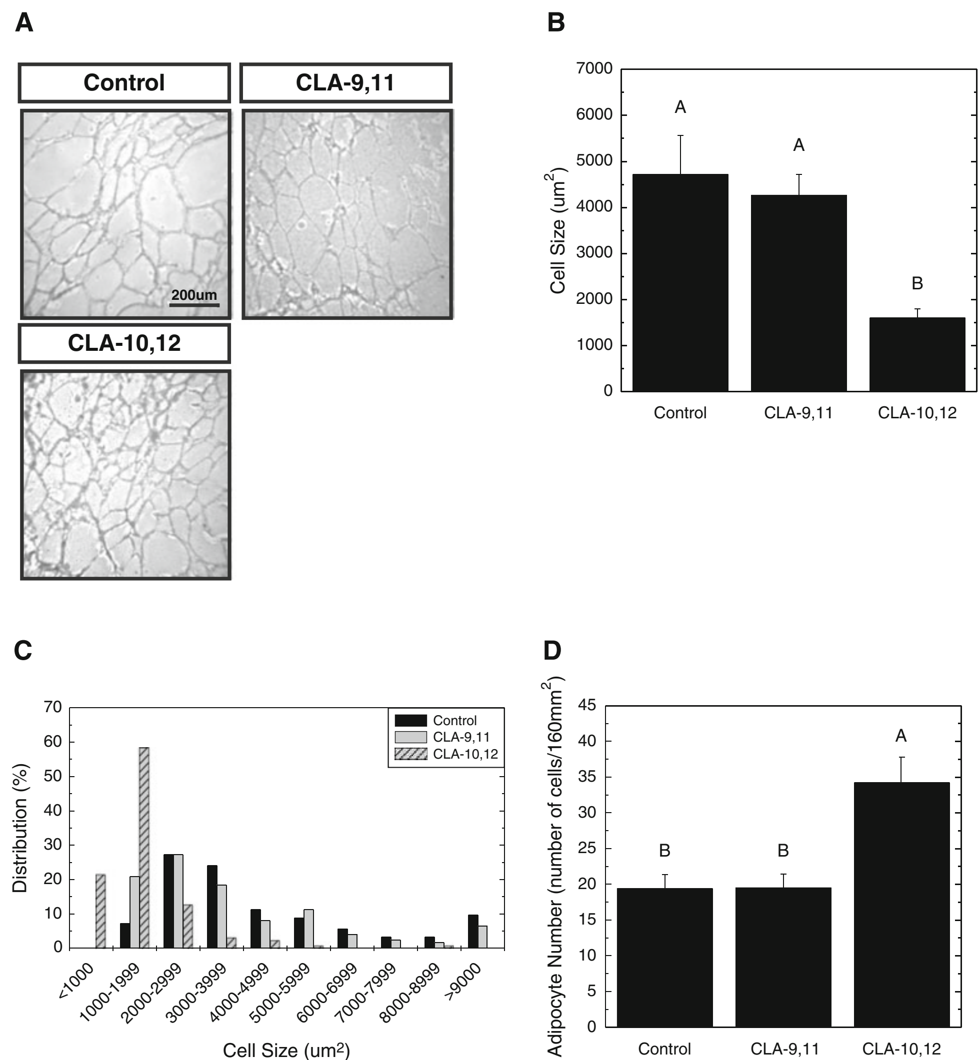

Fig. 1 Morphometry of epididymal adipose tissue from *fa/fa* Zucker rats fed CLA isomers for 8 weeks (n = 5/group). Adipocyte size (**a**) was quantified for each treatment group and cell size (**b**) is reported in μm^2 for the overall mean ± SEM. The distribution of adipocyte size (**c**) in each treatment group is also shown. Number of adipocytes (**d**) for each treatment group is reported as overall mean number of cells ± SEM per 160 mm^2. Means with different letters are significantly different ($P \leq 0.05$) as determined by Duncan's multiple range test. Control, *fa/fa* Zucker rats fed 0% CLA; CLA-9,11, *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12, *fa/fa* Zucker rats fed 0.4% t10,c12-CLA

53–63% of cells in the 2,000–5,000 μm^2 range and more than 25% of the cells were in excess of 5,000 μm^2 (Fig. 3c). The group receiving the t10,c12-CLA isomer also had 43% more adipocytes compared to the control and c9,t11-CLA groups (Fig. 1d).

Adipokines in Adipose Tissue

It is suggested that adipocyte size alters the production of adipokines [3–5] and because the t10,c12-CLA isomer reduced adipocyte size we decided to explore the adipokine status in the adipose tissue. Unexpectedly, the AngII precursor angiotensinogen was not changed at the gene (Fig. 2a) or protein level (Fig. 3a) in the adipose tissue of the rats fed CLA isomers. There was also no change in AngII receptors (Figs. 2e, f, 3b). Similarly, TNF-α, IL-6 and IL-10 mRNA were unchanged with CLA isomers (Fig. 2b–d). Likewise, protein levels of TNF-α, IL-6 and IL-10 were similar among all groups (Fig. 3c–e). Because macrophage infiltration occurs in obesity and contributes to the pro-inflammatory state of adipose tissue [5], the macrophage marker F4/80 was examined. None of the diets influenced the levels of macrophages within the adipose tissue (Fig. 4a).

NF-κB Activation

Given that NF-κB regulates many genes involved in inflammation [11], we next examined the levels of phosphorylated NF-κB and its promoter binding activity in epididymal adipose tissue. Neither CLA isomer affected either NF-κB phosphorylation (Fig. 4b) or DNA binding (Fig. 4c).

Discussion

The current study has demonstrated that feeding t10,c12-CLA to obese *fa/fa* Zucker rats reduces epididymal adipocyte size and increases cell number. The effect of CLA

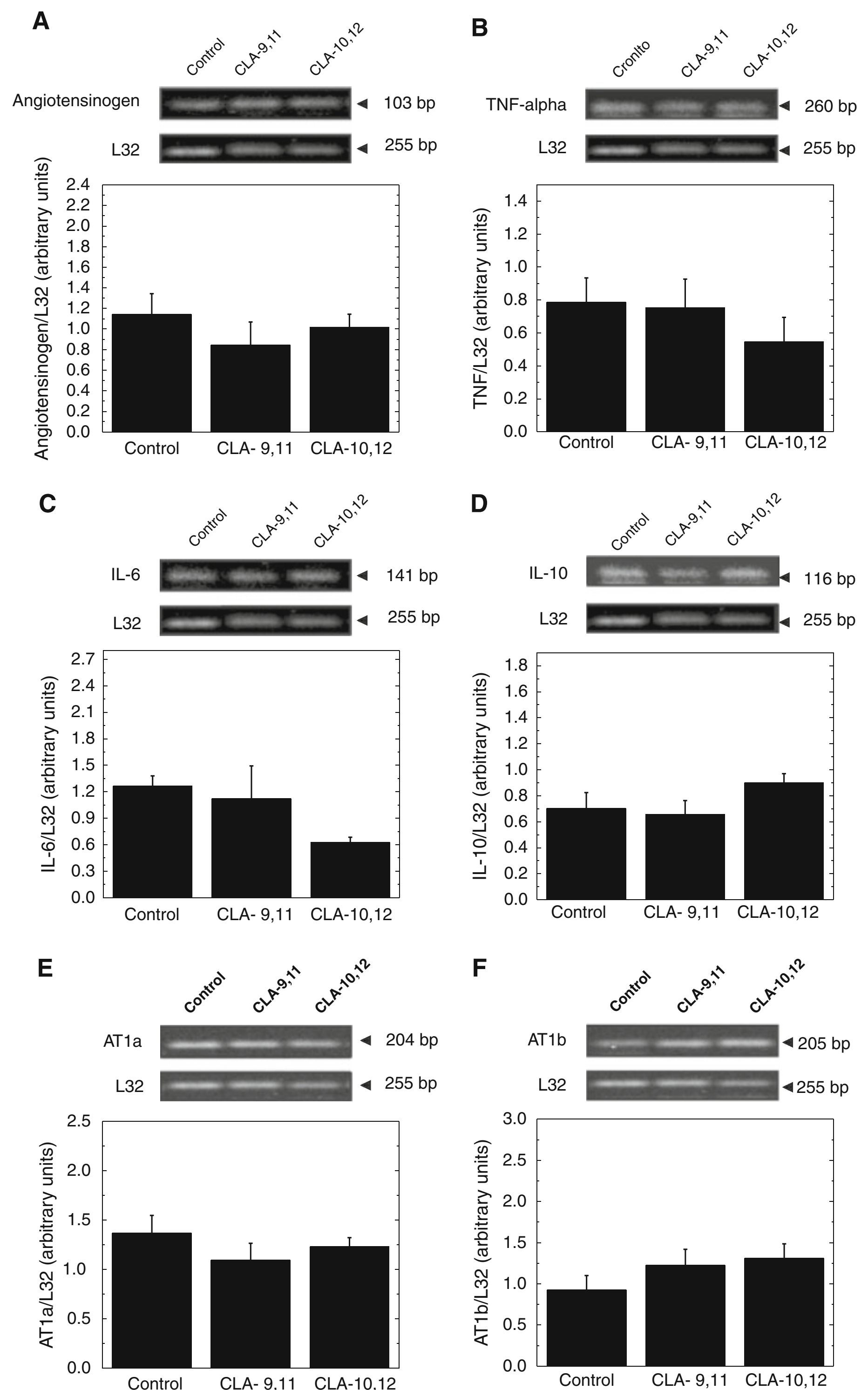

Fig. 2 Epididymal adipose tissue mRNA levels of angiotensinogen (**a**), TNF-α (**b**), IL-6 (**c**), IL-10 (**d**), AT1a (**e**) and AT1b (**f**) in *fa/fa* Zucker rats ($n = 6$/group). mRNA was analyzed using RT-PCR. The relative intensity of the bands was quantified by densitometry and normalized to that of ribosomal protein L32. Representative gels are shown for each plot. Data are presented as means $\pm$ SEM. No significant differences ($P > 0.05$) were detected by ANOVA. Control, *fa/fa* Zucker rats fed 0% CLA; CLA-9,11, *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12, *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; AT1a, AngII receptor type 1a; AT1b, AngII receptor type 1b; L32, ribosomal protein L32; bp, base pair

on adipocyte size in rats has previously been examined with either a mixture of CLA isomers [14, 26] or single isomers in a non-obese model [27]. Earlier work examining the effects of CLA mixtures on adipocyte size revealed that other types of fat in the diet may influence the effects of CLA [14, 26, 27]. For example, rats consuming diets based on a soybean oil background and supplemented with a mixture of CLA isomers have reduced cell size [14, 26], whereas the effects of CLA are lost when the background consists of saturated palm oil [27]. In the same study that used palm oil, isomer specific effects were also examined and the c9,t11-CLA isomer increased adipocyte size and

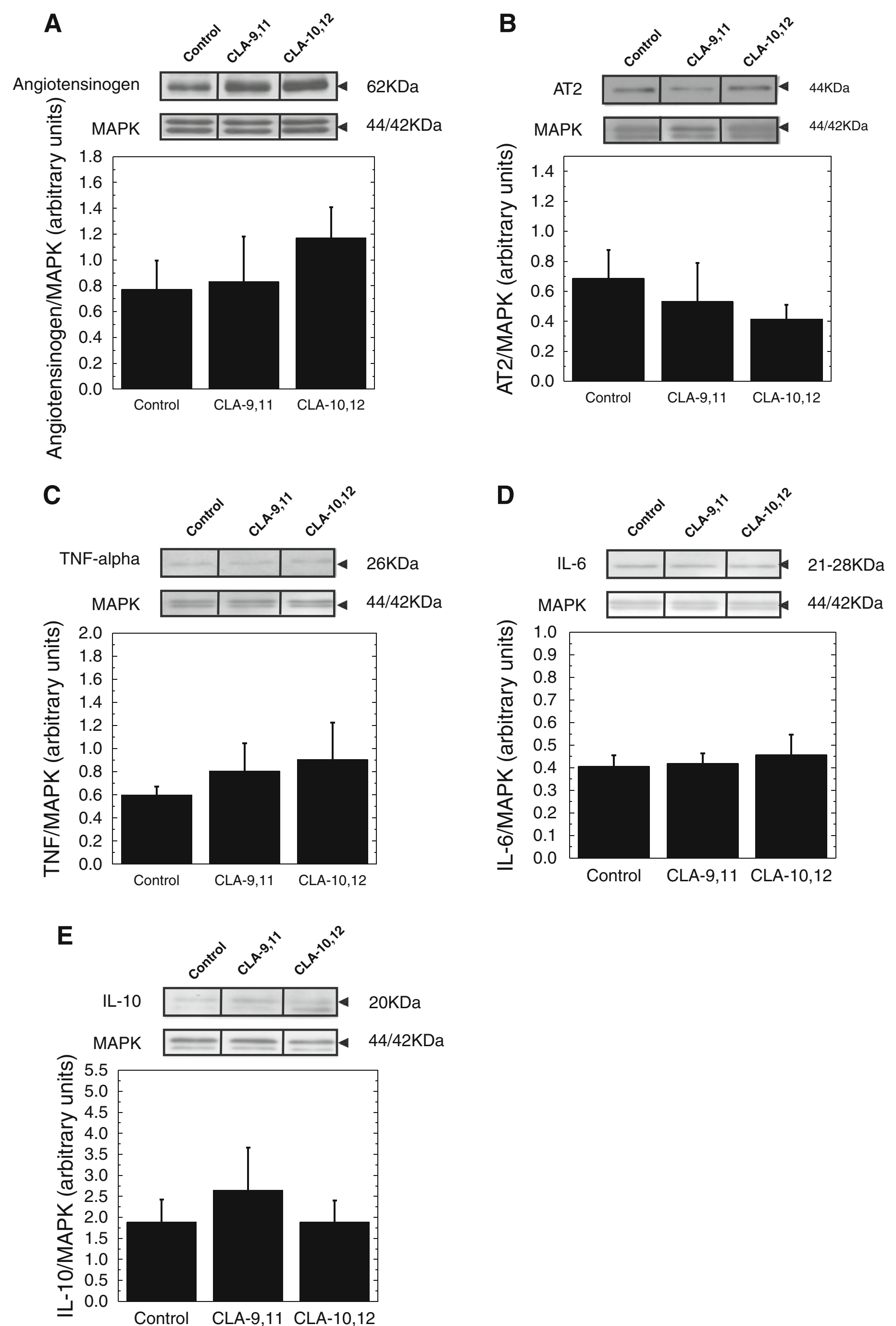

Fig. 3 Epididymal adipose tissue protein levels of angiotensinogen (**a**), AngII receptor type 2 (**b**), TNF-α (**c**), IL-6 (**d**) and IL-10 (**e**) in *fa/fa* Zucker rats ($n = 4$–6/group). Protein levels were measured using Western blot analysis. The relative intensity of the protein bands was quantified by densitometry and normalized to that of MAPK. Representative Western blots are shown for each plot. Data are presented as means ± SEM. No significant differences ($P > 0.05$) were detected by ANOVA. Control, *fa/fa* Zucker rats fed 0% CLA; CLA-9,11, *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12, *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; AT2, AngII receptor type 2

consequently decreased the number of adipocytes per unit of area in the inguinal and retroperitoneal depots of Wistar rats [27]. The increase observed with the c9,t11-CLA isomer was abolished when a mixture of CLA isomers was provided [27], suggesting t10,c12-CLA may have beneficial effects for reducing cell size. Lopes et al [27] also showed rats receiving the t10c12-CLA isomer had significantly more adipocytes per unit area compared to the group receiving the c9,t11-CLA isomer. In agreement with this hypothesis, our study demonstrated that the t10,c12-CLA isomer is responsible for reducing cell size and increasing adipocyte number. Additionally, because earlier work in *fa/fa* Zucker rats fed a mixture of CLA isomers demonstrated that reduced adipocyte size was associated with altered adipokines, we wanted to test if the beneficial changes in adipocytes size observed with the t10,c12-CLA isomer also produce changes in local adipose RAS and cellular adipokine status.

Evidence suggests that obesity-related complications may be due in part to the activation of a local RAS in

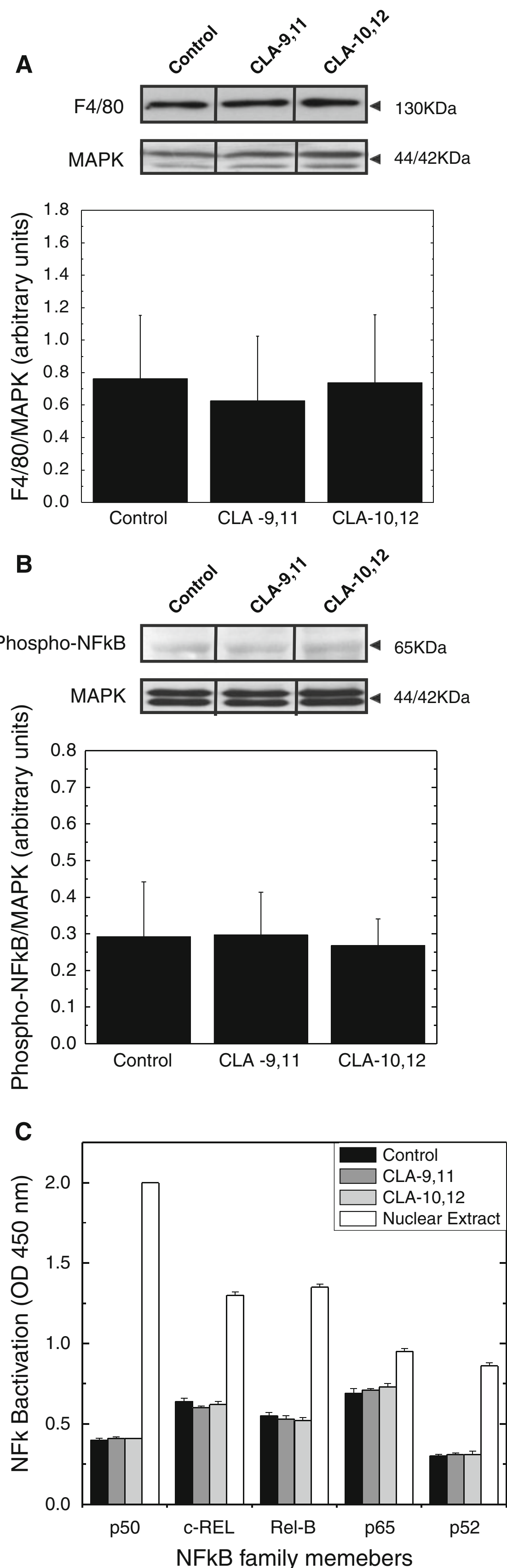

Fig. 4 Epididymal adipose tissue F4/80 (**a**), phosphorylated NF-κB levels (**b**) and NF-κB binding activity (**c**) in *fa/fa* Zucker rats ($n = 6$/group). Protein levels were measured using Western blot analysis. Relative intensity of the protein bands was quantified by densitometry and normalized to that of MAPK. A representative Western blot is shown. Activation of NF-κB was measured with TransAM ELISA kits (Active Motif). Data are presented as means ± SEM. No significant differences ($P > 0.05$) were detected by ANOVA. Control, *fa/fa* Zucker rats fed 0% CLA; CLA-9,11, *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12, *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; nuclear extract, positive control

adipose tissue [9, 28, 29], however, in the current study, no significant differences were detected in adipose angiotensinogen levels or AngII receptors of CLA fed rats. To our knowledge this is the first report describing the effect of CLA isomers on AngII receptors. In contrast, previous work showed OLETF rats fed the t10,c12-CLA isomer had suppressed angiotensinogen gene expression in peri-renal adipose tissue [16]. Furthermore, Hainault et al. [30] showed angiotensinogen levels were greater in inguinal adipocytes isolated from obese compared to lean Zucker rats. Perhaps assessment of angiotensinogen mRNA or protein levels in different adipose depots or different models may be responsible for the varying results. Epididymal adipose tissue was investigated in this study because alterations in adipokine levels in epididymal adipose tissue have been noted in this animal model [14]. We measured angiotensinogen protein levels in epididymal adipose tissue as well as peri-renal adipose tissue (data not shown), however, we were unable to detect any dietary effects on angiotensinogen levels in either depot. Additionally, it may be that a reduction in fat mass is required in rats to achieve changes in angiotensinogen mRNA [31, 32].

Despite observing changes in adipocyte size, we did not note any changes in adipokine status or macrophage inflammation in the epididymal adipose tissue. However, it is still possible that the inflammatory mediators produced in liver, spleen, or other tissues could affect changes observed with CLA supplementation. Gollisch et al. [33] examined exercise as a means to reduce cell size and inflammatory cytokines in Sprague Dawley rats, however, results showed that maintenance of a small adipocyte size by exercise did not alter IL-6 or TNF-α mRNA compared to sedentary rats with increased adipocyte size. Thus, lifestyle interventions aimed at altering adipocyte size to improve inflammatory adipokine status of rats require more investigation before conclusions can be drawn. In contrast, studies in mice and isolated human adipocytes have clearly demonstrated that the t10,c12-CLA isomer increases mRNA levels of IL-6 and TNF-α in adipose tissue [17, 34]. Unlike the rat model used in the current study, mice

(irrespective of their metabolic state) also display increased macrophage infiltration when given t10,c12-CLA or a mixture of CLA isomers [17, 35–37]. This study thus adds to the literature that has demonstrated differences in the inflammatory response between mice and rats when given the t10,c12-CLA isomer.

Previous work using cell culture models suggests that CLA isomers may be able to modulate NF-κB activity [18, 38, 39], thus to our knowledge this is the first study to examine phosphorylation and binding activity of NF-κB in adipose tissue after dietary intervention. Our results in obese rats show no effect of either CLA isomer on NF-κB phosphorylation or binding activity, however, these results were not unexpected given that levels of the inflammatory adipokines examined were unchanged by the treatments. In contrast to the current study, isolated human adipocytes treated directly with t10,c12-CLA isomer have increased TNF-α and IL-6 mRNA and this was associated with an increase in IκBα phosphorylation and NF-κB binding activity [18]. Likewise, increased NF-κB binding activity was demonstrated in human umbilical vein endothelial cells treated with either the c9,t11-CLA or t10,c12-CLA isomer [38]. In a mouse macrophage cell line (RAW264.7), however, a mixture of CLA isomers reduced phosphorylated IκBα and NF-κB binding activity [39]. Additionally, a significant increase in NF-κB activity is observed in murine myotubes treated with t10,c12-CLA isomer but not murine myoblasts [40]. Thus, it appears that differing results occur not only between obese rat and mouse models but also different cell types, emphasizing the need for more research in this area. Future work is necessary to determine cell and species-specific effects of CLA isomers, as well as determine if therapeutic strategies that reduce adipocyte size can directly alter adipokine production and secretion.

In conclusion, this study demonstrates that t10,c12-CLA is the isomer responsible for the reduction in adipocyte size that was observed in an earlier study using a mixture of CLA isomers in *fa*/*fa* Zucker rats [14]. The reduction in adipocyte size, however, did not alter components the local adipose RAS such as angiotensinogen and AngII receptors, or associated adipokines such as TNF-α, IL-6 and IL-10. Furthermore, CLA isomers have no influence on macrophage infiltration or NF-κB activity in the adipose tissue of obese Zucker rats. Therefore, further work is needed to determine if there are any functional changes in the adipocyte as a result of reduced cell size via t10,c12-CLA isomer treatment.

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Conflict of interest None.

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