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10E12Z CLA alters adipocyte differentiation and adipocyte cytokine expression and induces macrophage proliferation☆

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Abstract

The *trans*-10, *cis*-12 (10e12z) conjugated linoleic acid (CLA) isomer of CLA is responsible for loss of lipid storage or adipose tissue *in vitro* or *in vivo*. This isomer also induces inflammatory signaling in both mouse and human adipocytes *in vitro*. However, when these events occur and whether they are significant enough to affect other cell types are unclear. In these experiments, the 3T3-L1 cell line has been used to examine the interaction between inflammatory signaling and decreased differentiation or lipid storage induced by 10e12z CLA. In assays measuring both lipid accumulation and gene expression, differentiating 3T3-L1 cells exhibit concurrent induction of inflammatory signaling, as measured by cyclooxygenase-2 expression, and a decrease in adipocyte marker gene expression. Furthermore, in fully differentiated adipocytes, as identified in microarray assays and confirmed with real-time polymerase chain reaction, 10e12z CLA also significantly affected expression of both matrix metalloprotein-3 (MMP-3), collagen VI α 3 ColVI alpha 3 (VIα3) and the cytokine epiregulin, demonstrating that the effects of 10e12z broadly impact adipocyte function. In agreement with other experimental systems, 10e12z CLA inhibited RAW 264.7 cell proliferation; however, in response to adipocyte-conditioned media, 10e12z-CLA-treated adipocytes induced proliferation of this cell line, suggesting that the effect of 10e12z CLA is context dependent. These results are largely consistent with the known activation of the inflammatory mediator nuclear factor-κB in adipocytes *in vitro* and *in vivo* by 10e12z CLA treatment and demonstrate that adipose is an important target tissue of this isomer that impacts other cell types.

Keywords: Inflammation; 3T3-L1 adipocytes; Paracrine effects; Extracellular matrix; Cyclooxygenase

1. Introduction

The adipocyte plays an integral role in energy homeostasis of the whole organism. Adipocytes are important in regulating energy metabolism through the release of a broad array of endocrine-acting factors [1]. A prominent example of this ability is the secretion of adiponectin, which modulates energy utilization in distant tissues including liver and muscle [2–4]. Adipocytes also contribute to the detrimental symptoms of metabolic syndrome and diabetes as they are competent to initiate inflammatory signaling leading to macrophage infiltration and obesity-associated inflammation and insulin resistance [1,5]. Similar to leukocyte-induced inflammatory signaling, adipose-inflammatory signaling through cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 results in increased macrophage infiltration that further enhances cytokine and chemokine production [6,7] and plays a specific role in increased insulin resistance [8]. Maintaining proper adipocyte function is an important health goal, and dietary and environmental chemicals or diseases that reduce adipocyte function are of great concern [9,10].

Conjugated linoleic acid (CLA) isomers have gained notoriety for their anti-inflammatory actions in multiple model systems and antiproliferative effects in cancer cell lines [11–13]. Conjugated linoleic acid isomers exhibit a variety of anti-inflammatory mechanisms *in vitro* including inhibition of nuclear factor (NF)-κB transcriptional activity in several cell lines [14–16]. The *trans*-10, *cis*-12 (10e12z) CLA isomer exhibits anti-inflammatory effects in macrophage cell lines, while in fully differentiated adipocytes, this isomer decreases lipid storage and increases insulin resistance [14]. These effects are also seen in mice and humans, especially in overweight or obese subjects [17]. Mice fed 10e12z CLA develop hepatic steatosis and increase adipose expression of inflammatory markers such as IL-6 and IL-8 as well as decrease expression of adiponectin [18–20], suggesting that the effects of 10e12z CLA on adipose are directly opposed to their effects in other tissues [14]. *In vitro* studies illustrate that 10e12z CLA reduces insulin sensitivity of adipocytes and reduces glucose uptake while increasing rates of lipolysis [21]. These events lead to an increase in circulating glucose and lipotoxicity in mice and humans [18,20,22] and have been associated with increased NF-κB activity and cyclooxygenase (COX)-2

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expression in fully differentiated human adipocytes [23–25]. These cell-specific effects are especially significant given that the effects on adipose are the most consistent effect of 10e12z CLA feeding in mice. Although decreased adipocyte differentiation occurs in adipocytes treated with 10e12z CLA [21,26,27] and has been proposed to be partially responsible for the decrease in body fat caused by 10e12z CLA [28], no specific inflammatory involvement has been demonstrated in differentiating adipocytes.

In these experiments, the effect of 10e12z CLA on differentiating and fully differentiated 3T3-L1 cells was studied. 10e12z CLA decreased the extent of 3T3-L1 adipocyte differentiation when treated throughout the differentiation period. These changes became evident only midway through the differentiation protocol and occurred concurrent with increased COX-2 expression, a marker of NF-κB activity. Analysis of gene expression of fully differentiated cells demonstrated multiple changes in adipocyte function and substantiated the involvement of NF-κB in altering adipocyte function in response to 10e12z CLA. Finally, the response of 10e12z-CLA-treated adipocytes was sufficient to impact other cell types as conditioned media from 10e12z-CLA-treated adipocytes affected proliferation of the macrophage cell line RAW264.7, suggesting that increased macrophage numbers described *in vivo* [20] are due to the adipocyte-specific effect of this CLA isomer.

2. Materials and methods

2.1. Cells and cell culture 3T3-L1

Cells were purchased from ATCC (Manassas, VA, USA) and grown in high-glucose Dulbecco's modified Eagle's medium (HGDMEM; Sigma, Saint Louis, MO, USA) supplemented with 10% calf serum (Hyclone, Logan, UT, USA) and 100 U of penicillin and 100 μg/ml of streptomycin (Invitrogen). 3T3-L1 fibroblasts were differentiated by allowing the cells to grow to confluency (Fig. 1). Two days after confluency, the cells were initiated to differentiate by changing the serum source to fetal bovine serum (FBS) and supplementing with 1 μg/ml insulin, 1 μM dexamethasone and 100 μM 3-isobutyl-1-methylxanthine (IBMX; MDI) for 2 days. Cells were either treated 2 days later with test compounds in HGDMEM containing 10% FBS plus 1 μg/ml insulin during differentiation experiments or were refed every 2 days with HGDMEM with 1 μg/ml of insulin for 10 days for experiments with fully differentiated adipocytes. After 10 days, BSA-treated controls all contained large lipid droplets and numerous small lipid droplets. Therefore, 10 days was considered full differentiation. In some instances, the BSA controls did not differentiate fully within the 10-day period; in these cases, the cells were not analyzed. Other chemicals used included rosiglitazone (rosi; Cayman Chemical), *cis*-9, *trans*-11 (9z11e) CLA and 10e12z CLA (Matreya, Pleasant Gap, PA, USA). Linoleic acid (LA) and all other chemicals were from Sigma (Saint Louis, MO, USA).

2.2. Fatty acid conjugates

10e12z CLA is nearly insoluble in aqueous solutions; therefore, all fatty acids were conjugated to fatty-acid-free BSA. This was accomplished following established protocols [29], maintaining a molar ratio of 4:1 (fatty acid:BSA). Fatty acid stocks and conjugates were stored at −20°C under argon. The resulting conjugates were tested for endotoxin contamination using the Limulus Amebocyte Lysate assay (LAL, E-Toxate, Sigma). In all cases, the conjugates contained <0.5 EU/ml endotoxin.

2.3. Quantitative oil red O staining

3T3-L1 cells were differentiated in 24-well plates as described above. After the appropriate treatment period, the cells were washed twice in phosphate-buffered saline. The cells were fixed, stained and extracted, and neutral lipid was quantified spectrophotometrically at 510 nm as previously described [30].

2.4. RNA analysis

Cells were lysed in Tri-reagent (Sigma), and total RNA was extracted following the manufacturer's protocol. One microgram of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (ABI, Foster City, CA, USA). Complementary DNA was diluted 1:200, and real-time polymerase chain reaction (PCR) was conducted with an ABI 7000 thermal cycler using Sybr Green PCR Master Mix (ABI). Gene specific primers were designed using Primer Express software (ABI) to span introns where possible. Primer sequence information can be found in the supplementary online materials. All messenger RNA (mRNA) expression data were corrected for 18s ribosomal RNA (rRNA) expression prior to analysis. Data are presented as the fold change relative to the single most appropriate treatment or time point in the study.

2.5. Microarray hybridizations and analysis

The Mouse Genome Oligo Set, Version 2, was purchased from Operon Technologies and printed onto amino-silane-treated slides using GeneMachines Omnigrid (San Carlos, CA, USA) at the Penn State University DNA Microarray Facility. Total RNA from fully differentiated 3T3-L1 adipocytes treated with either 50 μM 10e12z CLA or 50 μM LA was isolated by Tri-reagent (Sigma-Aldrich) according the manufacturer's instructions. Total RNA (1 μg) was amplified and labeled for two colored arrays using the Amino Allyl MessageAmp II aRNA Amplification Kit (ABI) and the Cye Dye Post Reactive Labeling Kit (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's instructions. The arrays were then blocked, hybridized with amplified RNA and washed following previously reported protocols [31]. Arrays were scanned with a GenePix 4000A scanner (Axon Instruments Inc., Foster City, CA, USA), and image intensity information was collected with Genepix 3.0 software (Axon Instruments). Data from Genepix files were filtered to remove bad spots and spots that were not significantly different ($P \leq .001$) from background. After filtering the data set, ratio data from approximately 12,000 genes was collected and normalized using lowess normalization and standard deviation regularization within a slide by Midas 2.16 from the TM4 suite [32]. Significantly regulated genes were determined using Statistical Analysis of Microarrays [33] with a median false discovery rate equal to 13.5% Delta of 0.323 as implemented in TM4. Using this approach, 329 genes, or 3%, were found to be increased by 10e12z CLA, while less than 1%, or 58 genes, were decreased by 10e12z CLA. These data sets were uploaded either together or independently into The Database for Annotation, Visualization and Integrated Discovery (DAVID) [34] to interpret the resulting gene lists by functional annotation, with a P value ≤.05 considered significant.

2.6. Proliferation assays

Treatments were prepared in HGDMEM containing insulin and 0.1% heat-inactivated FBS. Media were conditioned in empty wells (unconditioned media; UCM) or in wells containing fully differentiated 3T3-L1 adipocytes (adipocyte conditioned media; ACM) for 26 h and stored at −80°C until the macrophages were ready to be treated. RNA was extracted from the differentiated adipocytes to validate gene induction of inflammatory marker COX-2.

RAW264.7 macrophages were plated in clear 96-well plates at 10,000 cells per well in HGDMEM containing 10% heat-inactivated FBS. Cells were allowed to recover overnight; then serum was starved in media containing 0.1% heat-inactivated FBS for 18 h prior to treatment. Adipocyte and unconditioned BSA, CLA and epiregulin treatments were randomized across the plate. A duplicate standard curve, treated with UCM containing BSA, was generated for quantification of the results.

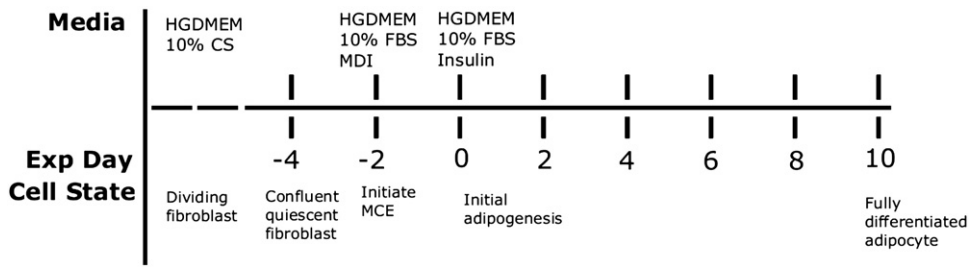

Fig. 1. Differentiation protocol for 3T3-L1 adipocytes used in this study. Timeline of differentiation used in these studies. The details are contained within the Materials and Methods section. CS, calf serum; MDI, IBMX, dexamethasone and insulin; Exp day; experimental day; MCE, mitotic clonal expansion.

Proliferation was determined after 72 h. Media were removed from the wells by aspiration. CellTiter-Glo Cell Viability Assay reagent (Promega, Madison, WI, USA) was diluted 1:3 in Opti-MEM, and 45 μl of diluted reagent was added to each well. Contents of the wells were transferred to a white plate, and luminescence measurements were taken using a Tecan GENios Pro (Männedorf, Switzerland) plate reader.

2.7. Statistical analysis

Experiments were typically set up in a completely randomized or a randomized complete block design. Experiments that used multiple treatments or time and treatment conditions were analyzed as factorial design. All data were analyzed for normalcy and homogeneity of variance prior to analysis of variance (ANOVA) using the GLM procedure in Minitab V. 14 (State College, PA, USA). Data that did not meet the assumptions of the ANOVA were transformed using the Box–Cox procedure as implemented in Minitab. Treatment groups that differ are identified in graphs with different letters or with an asterisk. Significant differences between multiple treatment groups were distinguished by Tukey's test with $P<.05$ considered significant. All data are represented in graphs as the mean (±S.E.M.).

3. Results

3.1. 10e12z CLA treatment during differentiation inhibits adipocyte specific gene expression and induces COX-2

The 10e12z CLA isomer inhibits adipose differentiation and induces NF-κB transcriptional activation in human adipocytes [23]. Cyclooxygenase-2 is also induced by 10e12z CLA in fully differentiated human adipocytes *in vitro* [24]. Inflammatory signaling induced by 10e12z may lead to inhibition of adipocyte function, but may also affect adipocyte differentiation. To identify whether 10e12z CLA inflammatory signaling also alters adipocyte differentiation, 3T3-L1 preadipocytes were treated every other day throughout the normal differentiation protocol with 10e12z CLA, BSA (vehicle control), the PPARγ agonist rosiglitazone (rosi) or isomeric controls LA or 9z11e CLA, and their effects on lipid storage and gene expression were compared relative to vehicle treatment. Preadipocytes treated continuously during differentiation with rosi demonstrated an increase in neutral lipid accumulation as measured by oil red O staining when compared to vehicle alone (Fig. 2A). The 18-carbon fatty acid controls, 9z11e CLA and LA, demonstrated enhanced or no change, respectively, in adipocyte neutral lipid storage relative to the BSA vehicle. Treatment with 10e12z CLA, however, resulted in a significant decrease in neutral lipid accumulation relative to all other treatments by the end of the differentiation period. This decrease was approximately 30% of BSA treatment alone and approximately 50% of rosi-treated cells. The effect of 10e12z CLA on differentiating 3T3-L1 cells was also detectible by analysis of adipocyte-dependent gene expression. 10e12z CLA treatment over the 10-day differentiation period dramatically decreased the mRNA levels of adiponectin, aP2 and C/EBPα to 5%, 15% and 30%, respectively, of the BSA control levels (Fig. 2B). These changes are consistent with the previously reported decreases in adipocyte phenotype and function caused by 10e12z CLA. Concurrently, cells differentiated in the presence of 10e12z CLA also expressed significantly increased levels of COX-2 (Fig. 2B). The induction of COX-2 was approximately threefold higher than the vehicle control BSA levels, which did not differ significantly from any of the other treatments. This result demonstrates that 10e12z treatment during 3T3-L1 preadipocyte differentiation markedly represses the adipocyte phenotype and induces the expression of the inflammatory marker COX-2.

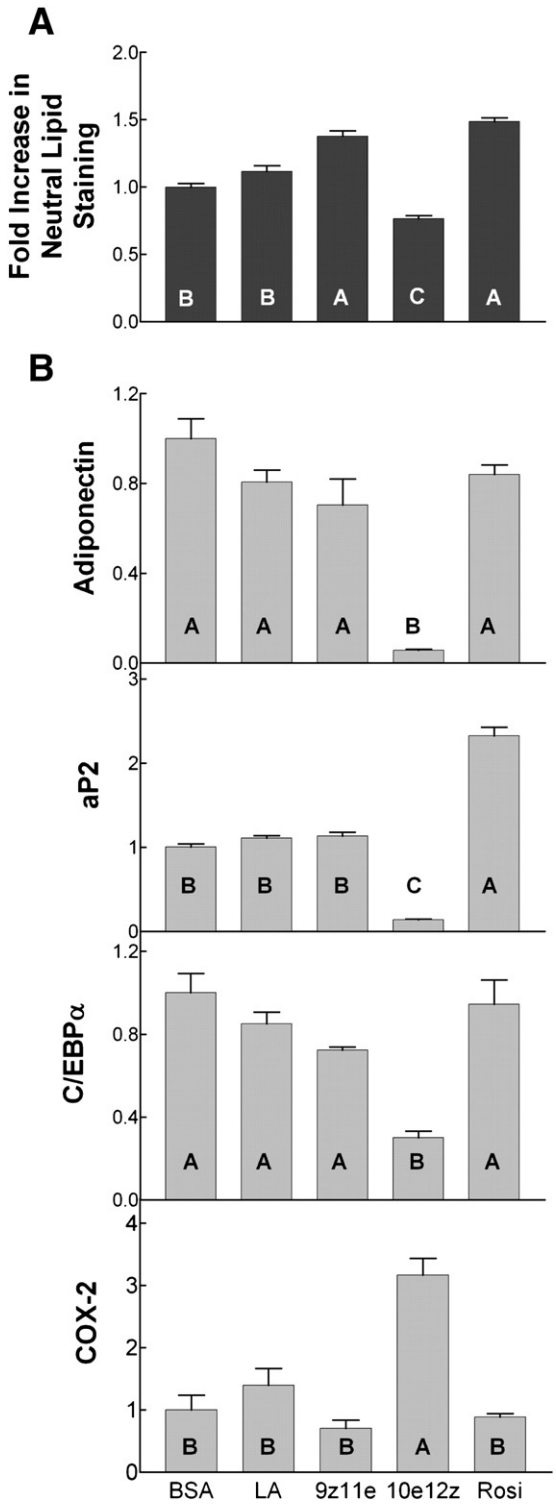

Fig. 2. 10e12z CLA reduces adipocyte differentiation but increases expression of COX-2 in differentiating 3T3-L1 cells. 3T3-L1 cells were grown and induced to differentiate as described in the Materials and Methods section. Two days after induction with MDI, the cells were treated with the indicated compounds every 2 days until day 8. On day 10, the cells were analyzed for changes in neutral lipid storage or gene expression. All data represent fold change over BSA vehicle treatments. (A) The PPARγ activator rosi (1 μM) and 9z11e CLA (50 μM) enhanced neutral lipid accumulation during differentiation, while treatment with 10e12z CLA (50 μM) reduced neutral lipid storage by approximately 30% from the BSA vehicle and by 50% from rosi (ANOVA $P<.05$, $n=4$). (B) Adiponectin, aP2 and C/EBPα expression relative to the BSA control, as measured by real-time PCR, was markedly decreased in 10e12z-CLA-treated adipocytes compared to all other treatments (ANOVA $P<.05$, $n=4$). However, cells differentiated in the presence of 10e12z CLA express significantly more COX-2 mRNA than other treatments ($P<.05$). Bars represent the mean±S.E.M. Treatment groups with different letters are significantly different from each other as determined by Tukey's post hoc analysis ($P<.05$).

3.2. CLA induction of COX-2 occurs concurrent with altered differentiation program

In human adipocytes, 10e12z CLA elicits an inflammatory response concurrent with a decrease in adipocyte character, and inhibition of NF-κB activity can restore that function [23]. To address whether 10e12z CLA elicits the same response in differentiating 3T3-L1 cells, the timing of sensitivity to 10e12z CLA during differentiation was examined by neutral lipid staining and gene expression analysis. Cells were treated with BSA, 9z11e or 10e12z CLA in the presence or absence of rosi. After 4 days of treatment in the presence of the PPARγ activator, cells stored significantly more lipid than those treated in the absence of rosi, although no differences in lipid accumulation were found between the fatty acid or vehicle treatments (Fig. 3A). By day 6, cells that were treated in the absence or presence of rosi with either 9z11e CLA or BSA accumulated more neutral lipid as determined by oil red O staining. Cells treated with 10e12z CLA, however, did not increase the amount of lipid accumulation, suggesting that the differentiating 3T3-L1 cells become sensitive to the presence of 10e12z CLA around days 4–6 independent of the presence of the potent PPARγ activator rosi.

The timing of the effect of 10e12z CLA on adipogenesis was also examined via analysis of aP2 mRNA expression in differentiating adipocytes. In the presence of rosi, expression of aP2 was induced 40-fold over the vehicle control as early as day 2 and continued to increase to about 80-fold by day 10 (Fig. 3B, top panel). In cells treated with the BSA vehicle, aP2 expression remained low until between days 5 and 10, when it increased by 30-fold over BSA at day 2, reaching approximately 50% of the rosi-stimulated cells on day 10. aP2 expression in cells treated with 10e12z CLA was not induced at any time point. In contrast to the expression of aP2, COX-2 was significantly induced by 10e12z CLA by day 5 and was elevated through day 10, an effect that was not seen in completely differentiated vehicle-treated controls (Fig. 3B) or in undifferentiated 3T3-L1 cells (data not shown). Together, these results demonstrate that 3T3-L1 cells measurably convert to an adipocyte phenotype between days 4 and 6 as measured by neutral lipid accumulation and after day 5 as measured by the expression of the PPARγ target gene aP2. In 3T3-L1 cells induced to differentiate but treated with 10e12z, these events are preceded by induction of inflammatory response as measured by COX-2 expression and as seen in completely differentiated adipocytes treated with 10e12z CLA, suggesting that a related mechanism may alter both the differentiation of adipocytes and the function of fully differentiated adipocytes [35].

3.3. Examination of pathways altered by CLA treatment of adipocytes: decreased lipid metabolism and increased cytokine signaling

Differentiating and fully differentiated adipocytes treated with 10e12z CLA both exhibit an inflammatory profile that may be responsible for the altered ability of these cells to differentiate or function normally. Therefore, global changes in gene expression of 10e12z-CLA-treated fully differentiated adipocytes were examined by microarray to examine the extent of inflammatory phenotype in these cells. Fully differentiated, day-10 adipocytes were treated for 12 h with 50 μM 10e12z CLA or LA as a fatty acid control. Treatment with 10e12z CLA significantly increased the expression of 329 genes and repressed expression of 58 genes relative to LA treatment as determined by SAM as implemented in TMEV (Supplementary Table 1). Consistent with a decrease in adipocyte function, the 10e12z-CLA-repressed gene set was significantly overrepresented in genes associated with lipid metabolism (Tables 1 and 2) including PPARγ co-regulator-1α, in agreement with a decrease of adipocyte function. Significantly repressed genes include C/EBPα and Spot 14, both of which are induced in the differentiating and mature adipocyte

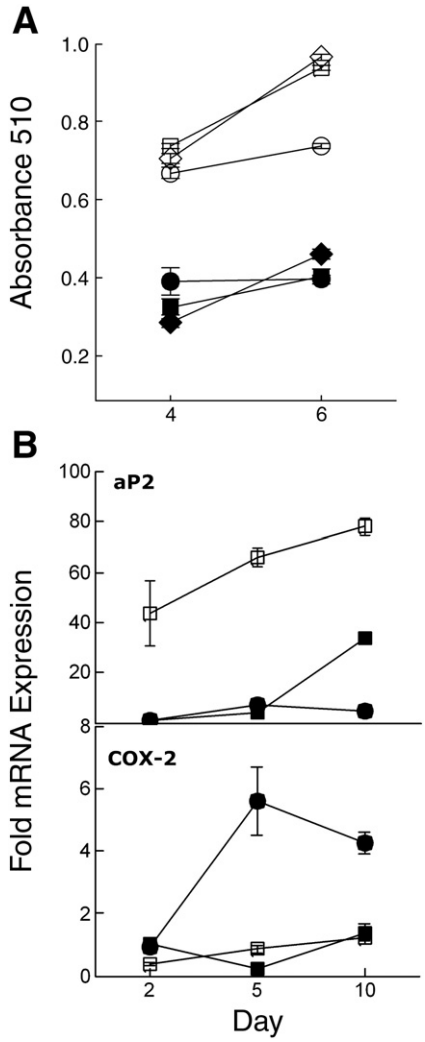

Fig. 3. Expression of COX-2 in differentiating adipocytes is concurrent with the onset of decreased adipocyte differentiation by 10e12z CLA. (A) 3T3-L1 fibroblasts were induced to differentiate and were treated on day 0 with (open symbols) or without 1 μM rosiglitazone (closed symbols) plus 75 μM 10e12z CLA (circles), 75 μM 9z11e CLA (diamonds) or 18.75 μM BSA (squares) every 2 days. Rosi-treated cells stored significantly more lipid than did cells treated in the absence of rosi. On day 4, cells treated without rosi did not differ from one another, nor did cells treated with rosi. However, when comparing between days 4 and 6, the cells treated with 10e12z CLA did not increase lipid storage, whereas both BSA and 9z11e CLA treatments did, irrespective of the presence of rosi (ANOVA $N=4$, $P<.05$). (B) Differentiating 3T3-L1 cells were treated 50 μM 10e12z CLA (circles) or 12.5 μM BSA (squares) every 2 days for 10 days. On the days indicated, cells were harvested and mRNA was extracted. Expression of aP2 mRNA (2B, upper panel) is significantly and rapidly increased by rosi, illustrating that PPAR γ activity is inducible as early as day 2. However, 10e12z CLA suppresses the increase that occurs in the presence of BSA alone ($N=4$, $P<.05$). In 10e12z-CLA-treated cells, expression of COX-2 mRNA (2B, lower panel) is induced approximately sixfold from days 5–10 over BSA- or rosi-treated cells ($N=4$, $P<.05$), concurrent with the onset of 10e12z CLA sensitivity as measured by lipid staining in differentiating 3T3-L1 cells.

[36,37]. Significant changes in expression of a subset of these genes were validated by real-time PCR (Fig. 4). The 10e12z-CLA-induced gene set was significantly overrepresented in genes associated with mitogen-activated protein kinase cascade, cytokine signaling and the toll-like receptor (TLR) pathways, consistent with an inflammatory state induced by 10e12z CLA (Table 1). This set of genes includes known 10e12z CLA targets such as ATF3, MCP-1, NF-κB and IL-6 (Table 2) [20,23,38,39].

Annotation analysis by DAVID indicated that a significant number of genes involved in the TLR signaling pathway were induced. TLR4 is

Table 1
Regulated processes

	Count in list	Percent	*P* value
Negatively regulated processes	58		
Aspartate metabolic process	2	3.2	.01
Cell development	9	14.3	.02
Cellular lipid metabolic process	6	9.5	.02
Lipid metabolic process	6	9.5	.04
Aspartate family amino acid metabolic process	2	3.2	.04
Negative regulation of cellular process	7	11.1	.05
Positively regulated processes	329		
MAPK signaling pathway	19	5.5	.00
Jak-STAT signaling pathway	9	2.6	.02
Toll-like receptor signaling pathway	7	2	.02
Chronic myeloid leukemia	6	1.7	.03
Acute myeloid leukemia	5	1.5	.04
Colorectal cancer	6	1.7	.04
Cytokine–cytokine receptor interaction	11	3.2	.04
Small cell lung cancer	6	1.7	.04
Prostate cancer	6	1.7	.04

activated by increased saturated fatty acids and was investigated further to determine if 10e12z CLA activated this receptor. TLR4 is expressed in a differentiation-dependent manner in 3T3-L1 adipocytes, and 10e12z significantly increased its expression (Supplemental Figure 1). However, 10e12z CLA did not activate a TLR4-dependent NF-κB reporter construct at doses that increase COX2 expression in 3T3-L1 adipocytes. Doses as high as 250 μM 10e12z CLA were unable to either activate this reporter construct or affect LPS-induced activation, demonstrating that although TLR4 is appropriately expressed in 3T3-L1 adipocytes, this receptor is not directly activated by 10e12z CLA treatment (Supplementary Figure 1).

Out of the 387 genes identified as being regulated by 10e12z CLA, 46 genes were functionally annotated as regulators of DNA-dependent transcription by DAVID ($P<.05$; Table 3). Real-time PCR analysis validated the expression changes of several of these factors including P53, Rel A, ATF3 and DDIT3 ($P<.05$, Fig. 4). Several AP-1 transcription factor members were identified as 10e12z CLA targets, of which JunB and FOSL1 were determined to be significantly induced by real-time PCR ($P<.05$, Fig. 4).

Table 2
Gene components of selected pathways identified by DAVID as significantly regulated

Pathway	Gene symbol	Mean ratio	S.E.M.
Cellular lipid metabolic process	Ppargc1a	−1.79	0.14
	Pik3r1	−1.99	0.33
	Nudt12	−2.28	0.15
	Sult1a1	−3.03	0.37
	Prlr	−3.2	1.03
	pnpla3	−3.22	0.69
Toll-like receptor signaling pathway	Cd14	2.84	0.23
	Spp1	2.85	0.54
	Jun	2.51	0.39
	Il-6	2.01	0.83
	Rela	1.72	0.32
	Myd88	1.6	0.47
	Nfkb2	1.47	0.23
Cytokine–cytokine receptor interaction	Ereg	5.08	0.97
	Bsf3	4.68	0.34
	Cxcl1	3.97	0.35
	Gdf15	3.9	0.14
	Areg	3.07	0.32
	Spp1	2.85	0.54
	Fgf21	2.56	0.59
	Il6	2.01	0.83
	Pdgfa	1.93	0.13
	Il1rn	1.86	0.24
	Hb-egf	1.78	0.2

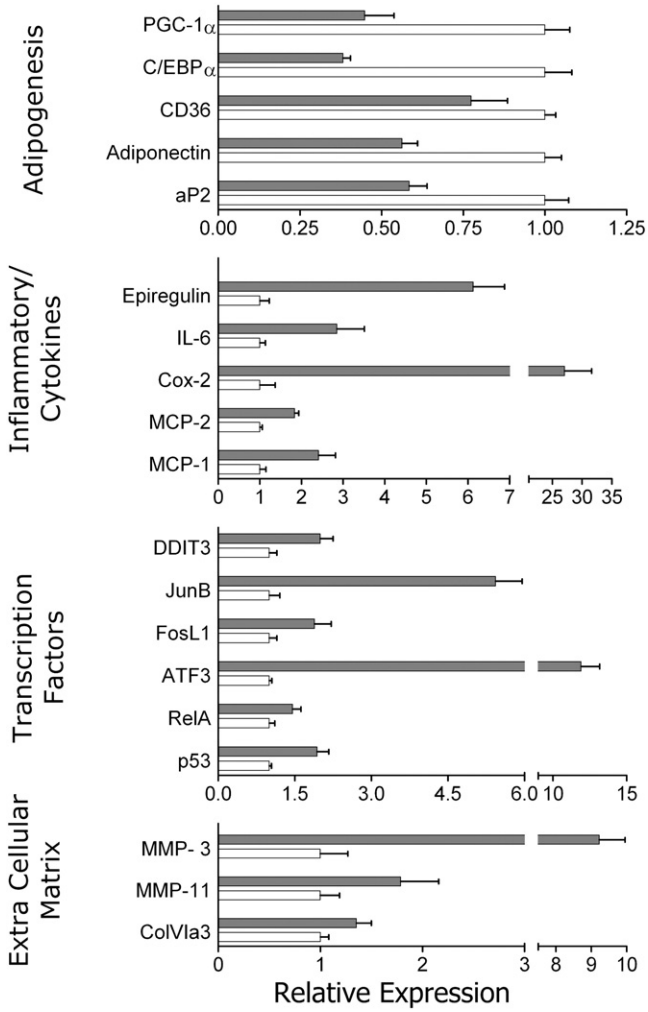

Fig. 4. Validation of gene expression profiles by real-time PCR. Expression of genes identified by microarray as being differentially regulated by 10e12z CLA (gray bars) and LA (white bars) in 10-day adipocytes after 12-h treatments with 50 μM 10e12z or LA was analyzed by real-time PCR. Data were standardized to 18s rRNA, and the fold change in expression relative to LA is depicted. All genes shown here differ significantly by treatment ($P<.05$, $N=3$-4).

The induction of several genes involved in extracellular matrix (ECM) remodeling was noted and validated (Supplementary Table 1 and Fig. 4). Collagen VIα3 (Col VIα3), matrix metalloproteinase (MMP)-3 and MMP-11 were validated by real-time PCR to be 10e12z-CLA-regulated genes in this system ($P<.05$, Fig. 4).

3.4. The effects of 10E12Z on macrophages are context dependent

The 10e12z isomer of CLA triggers an inflammatory response in 3T3-L1 adipocytes *in vitro* and in adipose tissue *in vivo* that, in turn, induces macrophage recruitment and activates inflammatory responses in adipose depots [35]. The adipocyte contribution to the effect of CLA on macrophage proliferation was evaluated using ACM. Medium containing BSA, 10e12z CLA or epiregulin, a mitogen highly induced by 10e12z CLA (Ereg; Fig. 4, Table 2), was incubated in the presence or absence of fully differentiated adipocytes for 26 h. This medium was placed on serum-starved RAW 264.7 macrophages, and proliferation was measured. Macrophage proliferation was unaffected

Table 3
Transcription factors regulated by 10e12z CLA as identified by DAVID

Name	Gene symbol	GenBank accession	Normalized ratio	S.E.M.
Activating transcription factor 3	Atf3	U19118	4.16	0.24
Aryl-hydrocarbon receptor	Ahr	–	3.24	0.13
Basic helix–loop–helix domain containing, class B2	Bhlhb2	AF010305	1.59	0.28
Bromodomain containing 2	Brd2	AF045462	1.55	0.25
CCAAT/enhancer binding protein (C/EBP), alpha	Cebpa	BC011118	−2.45	0.61
CCAAT/enhancer binding protein (C/EBP), delta	Cebpd	X61800	2.32	0.11
E26 avian leukemia oncogene 2, 3′ domain	Ets2	BC005486	2.07	0.39
Early growth response 1	Egr1	M22326	3.41	0.08
Early growth response 2	Egr2	X06746	2.41	0.18
Ets variant gene 5	Etv5	AK003461	1.83	0.2
Forkhead box C2	Foxc2	–	1.71	0.17
GATA binding protein 2	Gata2	AB000096	1.63	0.19
Heat shock factor 1	Hsf1	BC013716	1.42	0.26
High mobility group AT-hook 1	Hmga1	J04179	3.25	0.4
Inhibitor of DNA binding 3	Id3	M60523	2.15	0.03
Interferon activated gene 203	Ifi203	AF022371	2.72	0.74
Interferon activated gene 204	Ifi204	M31419	3.42	0.54
Jun oncogene	Jun	J04115	2.51	0.39
Jun proto-oncogene related gene d1	Jund1	J05205	2.08	0.07
Kruppel-like factor 16	Klf16	AF283891	1.98	0.23
Kruppel-like factor 2 (lung)	Klf2	U25096	2.79	0.12
Kruppel-like factor 4 (gut)	Klf4	U70662	2.14	0.29
Kruppel-like factor 5	Klf5	BC006646	2.27	0.6
Kruppel-like factor 6	Klf6	AK053584	2.69	0.46
Leucine-zipper-like transcriptional regulator, 1	Lztr1	AK083411	1.49	0.08
LIM homeobox protein 6	Lhx6	AB031040	1.62	0.27
Lin-9 homolog (C. elegans)	Lin9	AK012271	−2.49	0.24
MAD homolog 3 (*Drosophila*)	Smad3	AK048626	1.62	0.4
Myelocytomatosis oncogene	Myc	X01023	2.19	0.37
MyoD family inhibitor domain containing	Mdfic	AK048821	1.46	0.15
Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta	Nfkbib	U19799	1.64	0.43
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	Nfkb2	AF155373	1.47	0.23
Nuclear transcription factor, X-box binding-like 1	Nfxl1	AK005913	1.77	0.64
Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	Ppargc1b	AF453324	−2.68	0.5
Peroxisome proliferative activated receptor, gamma, coactivator-related 1	Pprc1	BC013720	1.82	0.21
PWP1 homolog (*S. cerevisiae*)	Pwp1	AK009972	1.93	0.68
SERTA domain containing 2	Sertad2	AB041541	1.68	0.06
Single stranded DNA binding protein 4	Ssbp4	AK004835	2.34	0.86
SRY-box containing gene 11	Sox11	AK012306	2.19	0.53
SRY-box containing gene 11	Sox11	AF009414	1.94	0.36
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	Smarca5	AF325921	1.54	0.41
TG interacting factor 1	Tgif1	X89749	4.69	0.52
Thyroid hormone responsive SPOT14 homolog (Rattus)	Thrsp	BG244447	−3.41	0.43
Transformation related protein 53	Trp53	BC005448	1.64	0.14
V-maf musculoaponeurotic fibrosarcoma oncogene family, protein K (avian)	Mafk	BC014295	2.92	0.26
V-rel reticuloendotheliosis viral oncogene homolog A (avian)	Rela	M61909	1.72	0.32
Zinc finger protein 113	Zfp113	BC052453	2.87	0.39

by BSA in either ACM or UCM, demonstrating that the ACM did not alter macrophage proliferation by itself (Fig. 5). 10e12z CLA in UCM reduced macrophage numbers relative to BSA by as much as 60% in UCM, while ACM generated from 10e12z-CLA-treated adipocytes strongly stimulated proliferation by nearly 200% relative to BSA ACM, demonstrating that the effect of 10e12z CLA on macrophage proliferation is context dependent. The role of 10e12z-CLA-responsive epiregulin in relation to macrophage proliferation was also examined. As with 10e12z CLA, epiregulin in UCM repressed macrophage proliferation by approximately 30%, while ACM generated from epiregulin-treated adipocytes stimulated macrophage proliferation by nearly 50%.

4. Discussion

In an isomer-specific manner, 10e12z CLA decreases adipocyte function and adipocyte differentiation [21,26,27,39]. Inflammatory signaling induced by 10e12z CLA activation of NF-κB is responsible for this effect in fully differentiated adipocytes [23,25], although a similar mechanism has not been identified in differentiating adipocytes. In differentiating adipocytes of either human or mouse origin, 10e12z CLA represses PPARγ-dependent gene transcription and TG accumulation. In isolated human stromal–vascular cells, inflammatory signaling is mediated by adipocytes and potentiated by preadipocytes [25]. Consistent with these findings, we demonstrate here that 3T3-L1 cells treated throughout differentiation exhibit decreased lipid storage and adipocyte-specific gene expression consistent with decreased PPARγ activity. Concurrently, these cells express increased COX-2 expression, consistent with the activation of NF-κB transcriptional activity.

As with human preadipocytes [25], 3T3-L1 fibroblasts do not respond to 10e12z CLA with an increase in inflammatory markers as determined by microarray [40] or examination of COX-2 expression (data not shown). An examination of the window of sensitivity of differentiating 3T3-L1 adipocytes demonstrated that treatment with 10e12z CLA for at least 4 days after induction to an adipogenic program was required prior to the observation of any change in the differentiation program. The first identifiable alteration in the differentiation program was the measurement of COX-2 at day 5 that occurred prior to measurable alterations in aP2 expression, a

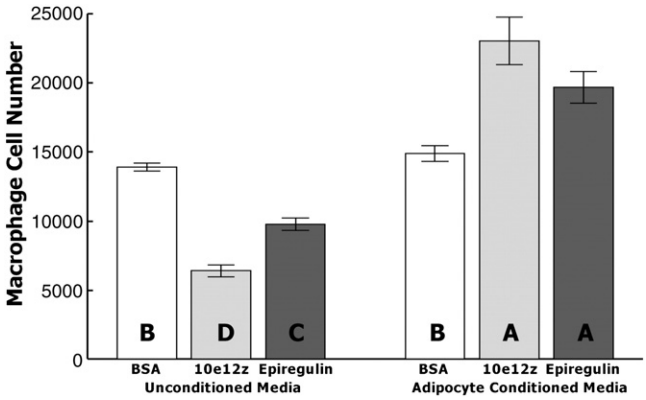

Fig. 5. Adipocyte-dependent effects on macrophage proliferation *in vitro*. The effects of 10e12z CLA on macrophages are context dependent and are altered by the activity of adipocytes secreted molecules. Medium treated with BSA, 10e12z CLA or epiregulin was conditioned in the presence of adipocytes or in empty wells. The conditioned media were administered to serum-starved RAW264.7 macrophages for 72 h. The effect of BSA-generated ACM was not different from UCM ($P>.05$). However the effects of epiregulin- or 10e12z-CLA-generated ACM are opposite to the effects of these treatments in UCM. Treatment with 10e12z CLA and epiregulin unconditioned media inhibits proliferation of RAW264.7 cells when compared to the BSA control ($P<.05$). Conversely, 10e12z CLA and epiregulin act through 3T3-L1 adipocytes and promote macrophage proliferation relative to BSA-generated ACM ($P<.05$). The two compounds result in a similar pattern of induction of RAW264.7 cell proliferation in ACM, suggesting that induction of epiregulin is among the key effectors of the 10e12z-CLA-induced inflammatory response in adipocytes. Bars represent the mean (±S.E.M.) of treatments. Bars with different letters are significantly different, Tukey's post hoc, $P<.05$, $N=6$.

marker of PPARγ activity. The increased COX-2 expression was also concurrent with increased lipid storage in control-treated adipocytes, suggesting, similar to the human model, that some differentiation of this cell culture model is required prior to sensitivity to this fatty acid.

Unlike other models of adipogenesis, 3T3-L1 cells can be completely differentiated to a multilocular stage in culture without the contaminating effect of other cell types [25], thus providing a homogenous population of cells. Gene expression studies of the 3T3-L1 adipocyte's response to 10e12z CLA demonstrate impacts on multiple functions, some of which have been corroborated by other studies. In epididymal adipose of obese M16 mice, a diet of 1% 10e12z CLA for 14 days decreased lipid metabolism and adipocyte differentiation, while increasing expression of apoptotic markers such as TNF-α and caspase-3 [41]. In C57BL/6J mice fed 0.5% 10e12z CLA, retroperitoneal (RP) adipose consistently decreases expression of genes involved in lipogenesis and beta-oxidation between 1 and 17 days, while mitochondrial uncoupling protein-1 and uncoupling protein-2 increase between 7 and 17 days [42]. 10e12z CLA does not affect feed intake, suggesting that increased energy expenditure was responsible for the adipocyte delipidation. Studies with 3T3-L1 adipocytes extended these findings as they are consistent with both CLA-fed C57BL/6J RP gene expression profiles as well as tunicamycin-induced endoplasmic reticulum stress [40]. More recently, the effects of 10e12z CLA on gene expression have been likened to those of the antidiabetic compound metformin [43]; both decrease the expression of genes involved in lipogenesis, and both expression profiles correlate with the UPR-inducing tunicamycin. The primary distinction in these profiles, however, is the inflammatory response induced by 10e12z CLA. In the present studies, 10e12z CLA increased expression of inflammatory genes and decreased the expression of genes involved in lipogenesis and adipocyte differentiation. However, these results also suggest a greater role for the transcription factor NF-κB.

Nuclear factor-κB regulates many important cellular functions including the inflammatory response and remodeling of the ECM [44–46]. Adipocyte function and differentiation are also regulated by the appropriate extracellular context [47,48]. Both MMP-3 and MMP-11 are involved in regulating adipose tissue development, as evidenced by the fact that adipose tissue in animals with either gene deleted experiences adipocyte hypertrophy [49,50]. Similarly, overexpression or addition of active MMP-11 to differentiated adipocytes can induce dedifferentiation [51]. Interestingly, collagen VIα3 is expressed by adipocytes *in vitro* and *in vivo*, and adipocyte-specific expression during mammary tumorigenesis leads to activation of β-catenin and other proliferative signals [52,53]. In addition, MMP-11 is responsible for cleavage and activation of Col VIα3, suggesting that the induction of these factors modulates the extracellular environment of adipose in concert [51] in response to 10e12z CLA. Along with the inflammatory cytokines regulated by NF-κB, these factors may also regulate adipose tissue function by altering the ECM milieu. These facts, along with the expression of inflammatory signals such as COX-2, suggest that the adipocyte function is directly inhibited by the induction of inflammatory signals by 10e12z CLA.

The specific signaling pathway(s) that activates NF-κB in response to 10e12z CLA is, as yet, undetermined. Multiple pathways are activated in adipocytes by 10e12z CLA [39,54], and many of these are likely activated secondarily through autocrine signaling mechanism from secreted cytokines such as IL-6, IL-8 and epiregulin in both preadipocytes and adipocytes [25,39]. These factors may also affect the behavior of surrounding cell types. Macrophage numbers are increased in adipose depots in mice fed 10e12z [20] either through activation and proliferation of adipose tissue macrophages or through recruitment of circulating monocytes into the adipose. Adipose-resident macrophages play a role in regulating insulin sensitivity and amplifying proinflammatory signaling of the adipocyte [8] and may play a role in the reduction of insulin sensitivity in response to 10e12z CLA *in vivo*. However, the effects of CLA differ distinctly in adipose and inflammatory models [14]. The anti-inflammatory properties of 10e12z CLA are well studied in macrophage models [55,56]. In human THP-1 macrophages, 10e12z CLA inhibits migration, COX-2 activity and macrophage-induced monocyte migration [57]. Therefore, the effect of 10e12z-CLA-treated adipocytes on RAW264.7 macrophage proliferation was examined *in vitro*. In this assay, unconditioned media containing 10e12z CLA repressed macrophage proliferation. When macrophages were treated with conditioned media from 10e12z-CLA-treated adipocytes, the cells increased their proliferation rate, suggesting that the response of these macrophages to 10e12z CLA is context dependent and that adipocytes provide these proliferative cues. As determined by gene expression profiling, there are many adipocyte-secreted factors that could be responsible for this effect. One of these factors is the potent mitogen epiregulin that was amongst the most highly induced cytokines detected. When adipocytes were treated only with epiregulin, the effects on macrophage proliferation were reproduced, suggesting a role for this cytokine in the macrophage response to 10e12z-CLA-treated adipocytes. This effect may be indirect, eliciting the secretion of other adipokines in an autocrine manner, as unconditioned media containing epiregulin did not elicit this effect but rather suppressed proliferation of RAW264.7 cells. In addition to epiregulin, other effectors of macrophage proliferation secreted from 10e12z-CLA-treated adipocytes include cytokines and growth factors, including IL-6, IL-8 [39] and molecules identified in Table 2, lipid mediators such as COX-2 products [14,24], ECM fragments [52] and potentially fatty acids released due to lipolytic action [58] of 10e12z CLA. Some or all of these factors are certain to be working in concert to induce macrophage proliferation to 10e12z-CLA-treated adipocytes.

The increased lean body mass spurred by CLA isomers is dependent upon the activity of the 10e12z CLA isomer [59]. The ability of 10e12z CLA to coordinately regulate the expression of ECM remodeling factors and inflammatory markers in adipose, along with

a significant number of cytokines, kinases and transcription factors, illustrates that the decreased lipid storage in adipocytes is a complex phenomenon that has repercussions on the whole tissue. While the decrease in adipocyte function is certainly, in part, due to modulation of PPARγ activity [60], our data suggest that this is an indirect or secondary response to the induction of NF-κB and inflammatory signaling. The complexity of the response of adipose to 10e12z CLA suggests that the promising and sought-after effects of decreased lipid storage are not likely to be separated from the negative effects of increased inflammatory signaling and decreased insulin sensitivity. As a tissue with specific impacts on multiple tissue types, the inflammatory effects incurred in adipose tissue in response to 10e12z CLA may readily be transmitted to other tissues that are not affected directly by 10e12z CLA.

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