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Journal of Nutritional Biochemistry 22 (2011) 656–664
Journal of Nutritional Biochemistry

Activated AMPK and prostaglandins are involved in the response to conjugated linoleic acid and are sufficient to cause lipid reductions in adipocytes☆

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Received 23 July 2009; received in revised form 18 December 2009; accepted 20 May 2010

Abstract

trans-10, *cis*-12 Conjugated linoleic acid (*t*10*c*12 CLA) reduces triglyceride levels in adipocytes. AMP-activated protein kinase (AMPK) and inflammation were recently demonstrated to be involved in the emerging pathways regulating this response. This study further investigated the role of AMPK and inflammation by testing the following hypotheses: (1) a moderate activation of AMPK and an inflammatory response are sufficient to reduce triglycerides, and (2) strong activation of AMPK is also sufficient. Experiments were performed by adding compounds that affect these pathways and by measuring their effects in 3T3-L1 adipocytes. A comparison of four AMPK activators (metformin, phenformin, TNF-α and *t*10*c*12 CLA) found a correlation between AMPK activity and triglyceride reduction. This correlation appeared to be modulated by the level of cyclo-oxygenase (COX)-2 mRNA produced. Inhibitors of the prostaglandin (PG) biosynthetic pathway interfered with *t*10*c*12 CLA's ability to reduce triglycerides. A combination of metformin and PGH2, or phenformin alone, efficiently reduced triglyceride levels in adipocytes. Microarray analysis indicated that the transcriptional responses to phenformin or *t*10*c*12 CLA were very similar, suggesting similar pathways were activated. 3T3-L1 fibroblasts were found to weakly induce the integrated stress response (ISR) in response to phenformin or *t*10*c*12 CLA and to respond robustly as they differentiated into adipocytes. This indicated that both chemicals required adipocytes at the same stage of differentiation to be competent for this response. These results support the above hypotheses and suggest compounds that moderately activate AMPK and increase PG levels or robustly activate AMPK in adipocytes may be beneficial for reducing adiposity.

Keywords: CLA; Metformin; Phenformin; Prostaglandin; Adipocyte; AMPK

1. Introduction

Dietary conjugated linoleic acid (CLA) causes dramatic reductions in adiposity in mice and in human adipocyte cultures [1–4]. An active isomer for reductions in white adipose tissue (WAT) is the *trans*-10, *cis*-12 isomer (*t*10*c*12 CLA [4]). The molecular responses to *t*10*c*12 CLA are remarkably diverse and include the integrated stress response (ISR [5,6]) or the unfolded protein response (UPR [7]), a nuclear factor kappa light chain enhancer of activated B cells (NF-κB)-mediated inflammatory response [3], mitogen-activated protein kinase (MAPK) cascades [8] and AMP-activated protein kinase (AMPK) [9,10].

AMPK is a central regulator of cellular energy levels that is activated by increases in the cellular AMP/ATP ratio, cellular processes [11,12] and *t*10*c*12 CLA in adipocytes [9] or mixed isomers of CLA in mice [10]. AMPK is a heterotrimeric protein, and activated AMPK requires phosphorylation at AMPKα threonine 172 [11,12]. Activated AMPK phosphorylates acetyl-CoA carboxylase (ACC) to inhibit fatty acid biosynthesis, and the amount of phosphorylated ACC provides a measurement of AMPK activity *in vivo* [13]. Drugs such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), metformin and phenformin directly or indirectly activate AMPK [11,14], while compound C is a potent inhibitor of AMPK. Either AICAR or metformin is sufficient to initiate an ISR in adipocytes [9,15], supporting a role for activated AMPK in this response. An ISR can activate NF-κB [16], and compound C blocks the ISR, activation of NF-κB and NF-κB-dependent inflammatory response [3] that are induced in *t*10*c*12 CLA-treated adipocytes [9], indicating these responses are AMPK dependent. Whether activation of AMPK is sufficient to explain the TG loss response caused by *t*10*c*12 CLA remains unresolved.

Abbreviations: ACC, acetyl CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; AA, arachidonic acid; ATF3, activating transcription factor 3; BSA, bovine serum albumin; CLA, conjugated linoleic acid; *t*10*c*12 CLA, *trans*-10, *cis*-12 conjugated linoleic acid; COX, cyclo-oxygenase; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; ISR, integrated stress response; JNK, c-Jun N-terminal Kinase; LA, linoleic acid; MCP-1, monocyte chemotactic protein-1; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NF-κB, nuclear factor κB; PG, prostaglandin; PLA2, phospholipase A2; PPARγ, peroxisome proliferator-activated receptor γ; TNF-α, Tumor Necrosis Factor alpha; TG, triglyceride; UPR, unfolded protein response; WAT, white adipose tissue.

☆ Supported in part by Agricultural Research Division Fellowships (to S. J. and Z. W.) and by the UNL Center for Biotechnology.

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doi:10.1016/j.jnutbio.2010.05.005

The inflammatory response has been proposed as a critical component of the triglyceride (TG) reduction that occurs in adipocytes exposed to *t*10*c*12 CLA [3–5,17–19]. NF-κB is required for the inflammatory response, and inhibition of NF-κB attenuates IL6 induction, the repression of GLUT4 and peroxisome proliferator-activated receptor γ (PPARγ) protein activity, and the reduced glucose transport that otherwise occurs in *t*10*c*12 CLA-treated human adipocytes [3]. Inhibition of NF-κB also attenuates the reduction in TG levels in *t*10*c*12 CLA-treated 3T3-L1 mouse adipocytes [9]. A key difference in the weak TG reduction that occurs in the presence of AMPK activator metformin and the more robust lipid loss mediated by *t*10*c*12 CLA is the stronger inflammatory response that occurs with *t*10*c*12 CLA [5,9]. The proinflammatory cytokine tumor necrosis factor α (TNF-α) is sufficient for lipid loss in adipocytes [20] and is moderately induced by *t*10*c*12 CLA in human adipocytes [3], but not in 3T3-L1 adipocytes [5]. PPARγ is a master regulator of adipogenesis and a key regulatory point for controlling inflammation in adipocytes [21]. PPARγ and NF-κB are mutually antagonistic, as PPARγ inhibits NF-κB transcriptional activity through transrepression [21], and NF-κB activity indirectly reduces PPARγ protein levels in the *t*10*c*12 CLA response [3].

*t*10*c*12 CLA increases prostaglandin (PG) biosynthesis in human adipocytes [6] and in mouse WAT [22]. PG biosynthesis is controlled in part by the rate-limiting release of arachidonic acid (AA) from the cell membrane by phospholipase A2 (PLA2). AA is then converted into PGH2, the precursor to the prostanoids, including the prostaglandins, by either cyclo-oxygenase (COX)-1 or by the inflammation-inducible COX2 [23]. PG production is increased in *t*10*c*12 CLA-treated adipocytes through increased activation of PLA2 [6], as measured by its increased phosphorylation, and increased levels of COX2 mRNA [5,6]. At least one member of the PG family, PGF2α, negatively affects adipocyte differentiation [24], but the functional role of increased PG biosynthesis in *t*10*c*12 CLA-treated adipocytes has not been established.

The interplay between AMPK activation and the inflammatory response, particularly its PG component, in causing the reduction of TG levels that occurs in *t*10*c*12 CLA-treated adipocytes is not currently well defined. The goal of this study was to test the following hypotheses: (1) a moderate activation of AMPK and an inflammatory response are sufficient to reduce triglycerides, and (2) strong activation of AMPK is also sufficient. Our experimental approach was to establish the possible involvement of specific pathways through chemical inhibitors, confirm that these pathways are activated by *t*10*c*12 CLA and then attempt to activate these pathways independently of *t*10*c*12 CLA to functionally assay their ability to reduce TG levels in adipocytes. Our rationale was that independent activation of the critical pathways should produce responses similar to those caused by *t*10*c*12 CLA and thereby provide additional support for the functional roles these pathways play. A potent activator of AMPK was analyzed for its similarity to *t*10*c*12 CLA in its ability to lower TG levels, in its whole genome transcriptional response and in its cell type-dependent induction of the ISR. The results of these studies support our hypotheses and provide new insights into the mechanisms utilized by *t*10*c*12 CLA to reduce TG levels in adipocytes.

2. Methods and materials

2.1. Reagents

Arachidonic acid, bovine serum albumin (BSA, >99% fat free), CP-24879, dexamethasone, ibuprofen, insulin, isobutyl-1-methylxanthine, metformin, naproxen, PGE_2, phenformin and TNF-α were from Sigma (St. Louis, MO, USA). AMPK inhibitor compound C was purchased from Calbiochem (San Diego, CA, USA). Antibodies to p-AMPK (α1 and α2), AMPK (α1 and α2), p-ACC (ACC1 and ACC2), ACC (ACC1 and ACC2), p-PLA2 and PLA2 were from Cell Signaling (Beverly, MA, USA). PGF2α, PGH2, SP600125 and U0126 were from Fisher (Pittsburgh, PA, USA).

2.2. 3T3-L1 cell culture and differentiation

3T3-L1 fibroblasts [25] were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 25 mM glucose and 10% bovine calf serum (Fisher) and differentiated as described [26]. In brief, 2 days after growing to confluence, the cells were induced to differentiate in DMEM containing 25 mM glucose and 10% fetal bovine serum (FBS; Fisher), 0.17 μmol/L insulin, 0.5 mmol/L isobutyl-1-methylxanthine and 1 μmol/L dexamethasone for 3 days. The medium was then changed to 10% FBS/DMEM containing 25 mM glucose and with 0.17 μmol/L insulin for another 2 days, at which time the cells were usually differentiated to at least 90% adipocyte, followed by basal 10% FBS/DMEM medium containing 25 mM glucose before initiating fatty acid treatments. All media contained 100,000 U/L penicillin and 172 μmol/L streptomycin (Invitrogen).

2.3. Fatty acid treatments

Fatty acids (>99%, Nu-check Prep, Elysian, MN, USA), either linoleic acid or *trans*-10, *cis*-12 CLA, were dissolved in 0.1 M KOH, diluted into fatty acid free (>99%) BSA in phosphate buffered saline at a 1:1 ratio (2 mmol/L BSA:2 mmol/L fatty acid), pH adjusted to 7.4 and added to the cultures containing 5.5- to 6-day post-differentiated 3T3-L1 adipocytes.

2.4. Western blots

Nuclear and cytosolic extracts were isolated based on the manufacturer's protocol (Active Motif, Carlsbad, CA, USA). Equal amounts of proteins were separated by SDS-PAGE, transferred to Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) and probed with the indicated antibodies. Enhanced chemiluminescence (Pierce, Rockford, IL, USA) was used for detection. Band intensities were determined from digital images from exposures in the linear range using imaging software (Quantity One, Bio-Rad). All Western blot analyses were repeated at least three times.

2.5. Quantification of TG content

Cell isolation and TG measurements were performed according to the manufacturer using TG reagent (T2449; Sigma) and free glycerol reagent (F6428; Sigma). Protein concentrations were measured using Bradford dye (Bio-Rad) to obtain results as micrograms of TG per milligram of protein.

2.6. Measurement of PGF2α

Cell culture concentrations of PGF2α were determined using culture media samples obtained immediately prior to cell harvest for TG analysis. PGF2α levels were measured by enzyme immunoassay (EIA) using the Correlate-EIA PGF2α Kit (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer's instructions. A standard curve was plotted using dilutions of a known concentration of the PGF2α standard, and sample concentrations were determined based on this standard curve. Data were derived from three independent experiments, with two replicates in each experiment.

2.7. Real-time PCR analysis of cDNA from mRNA

Total RNA was extracted by TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA (2 μg) was used for cDNA synthesis. Real-time PCR was performed by a Bio-Rad iCycler using iQ SYBR Green Supermix reagent (Bio-Rad). The primer sequences for MCP-1 and GAPDH were taken from Ref. [27], and all other primer sequences of genes analyzed by real-time PCR are shown in Supplementary Table 5. The cDNA levels were normalized to GAPDH, which showed no significant variation in microarrays between LA and *t*10*c*12 CLA treatments. Experiments were repeated three times, and each sample was analyzed using one RNA control and two replicates of each cDNA pool, and the relative amounts of each target cDNA and GAPDH were calculated using the comparative C_T method (according to the manufacturer's software (Bio-Rad) and to Ref. [28]). Cycle numbers were used to calculate gene expression levels in the linear amplification range, and qRT-PCR efficiency was close to 100%.

2.8. Microarray data analysis

RNA isolation and labeling of the 3T3-L1 adipocytes were performed as previously reported [5]. Results of the treatments of each of the three biological replicates were collected from Affymetrix GeneChip Mouse Genome 430 2.0 arrays via the Affymetrix GeneChip Operating Software (GCOS; Affymetrix, Santa Clara, CA, USA). The raw data for microarray results were normalized using the methods described by Wu et al. [29]. To identify differentially expressed genes between the control (linoleic acid) and other treatments, the linear models for microarrays (Limma [30]) package in R/Bioconductor was used, with Benjamini–Hochberg [31] adjusted *P* values. Those genes with an absolute log2 ratio of 2 or more (fourfold up- or down-regulated) and a Benjamini-Hochberg adjusted *P* value ≤.05 were deemed to be significant and analyzed further. The gene expression data has been deposited at the NCBI Gene Expression Omnibus with series number GSEXXXXX (access activated upon publication).

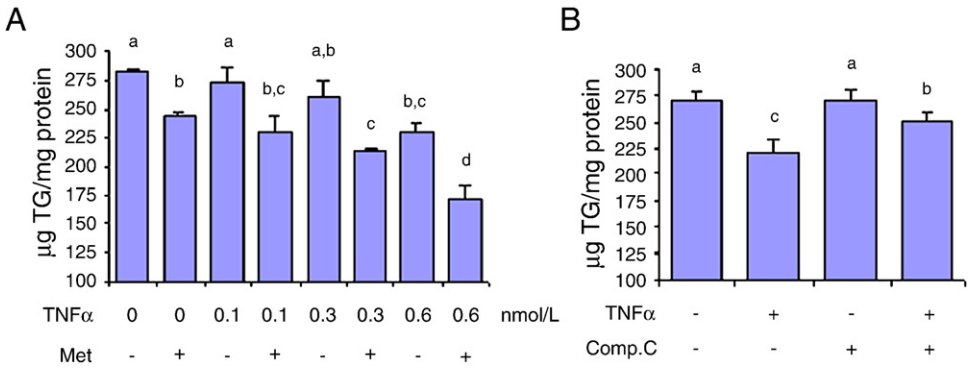

Fig. 1. TNFα increases the ability of metformin to reduce TG levels in adipocytes. (A) 3T3-L1 adipocytes were untreated or treated with either 2 mmol/L metformin (Met), the indicated amounts of TNF-α or combinations of these compounds, and TG levels were measured after 24 h. The effects of metformin and TNF-α were statistically significant, but their interaction was not. (B) 3T3-L1 adipocytes were either untreated or treated with 0.6 nmol/L TNF-α, 10 µmol/L compound C (Comp.C) or the combination, and TG levels were measured after 24 h. Their interaction was significant. Each bar represents the mean±S.E.M. (n=3) of three independent experiments.

2.9. Statistical analysis

Experiments represented in Figs. 1 through 6 were subjected to analysis of variance using fixed-effect statistical models. *F*-statistics, least-squares means and standard errors were calculated using SAS software (SAS Institute, Cary, NC, USA). The main effects or interactions were considered significant if $P \leq .05$, unless otherwise noted. For Fig. 1A, the main effects of TNF-α (0, 0.1, 0.3 or 0.6 nmol/L) and Met (− or +), and their interaction (TNF-α×Met) were analyzed. For Fig. 1B, the main effects of TNF-α (− or +) and compound C (− or +), and their interaction (TNF-α×Comp.C) were determined. The data presented in each panel of Fig. 2 were analyzed using a statistical model that included a single main effect. For Fig. 3, the main effects of CLA (− or +) and ibuprofen (− or +), naproxen (− or +) or CP-24879 (− or +), and their interaction (CLA×Ibuprofen, naproxen or CP-24879) were separately determined for both the TG and PGF2α experiments. For Fig. 4, the main effects of Met (− or +) and PGE_2 (− or +), PGF2α (− or +), PGH2 (− or +) or AA (− or +), and their interaction (Met × PGE_2, PGF2α, PGH2 or AA) were separately determined. For Fig. 5A, the main effects of CLA (− or +) and time (2, 4, 8 and 12 h), and their interaction (CLA×Time) were determined. For Fig. 5B and C, the main effects of CLA (− or +) and SP600125 (− or +) or U0126 (− or +), and their interaction (CLA×SP or U0126) were determined. For Fig. 6, the large range of the response values of ATF3 required both a log10 transformation and separating the data into a first group (Panel A) and second group (Panel B). The main effects of CLA (− or +), phenformin (− or +) and day (−2, 0, 1–5 days), and their interaction (CLA or Phen × days) were determined. Individual pairwise comparisons of least-squares means were calculated by least significant difference test. Data are presented as least-squares means±S.E.M. Means not sharing a common superscript differ significantly ($P \leq .05$), unless otherwise noted.

3. Results

Our hypothesis, based on prior use of chemical inhibitors, was that moderate activation of AMPK and an inflammatory response are sufficient to reduce TG levels in adipocytes. This was tested

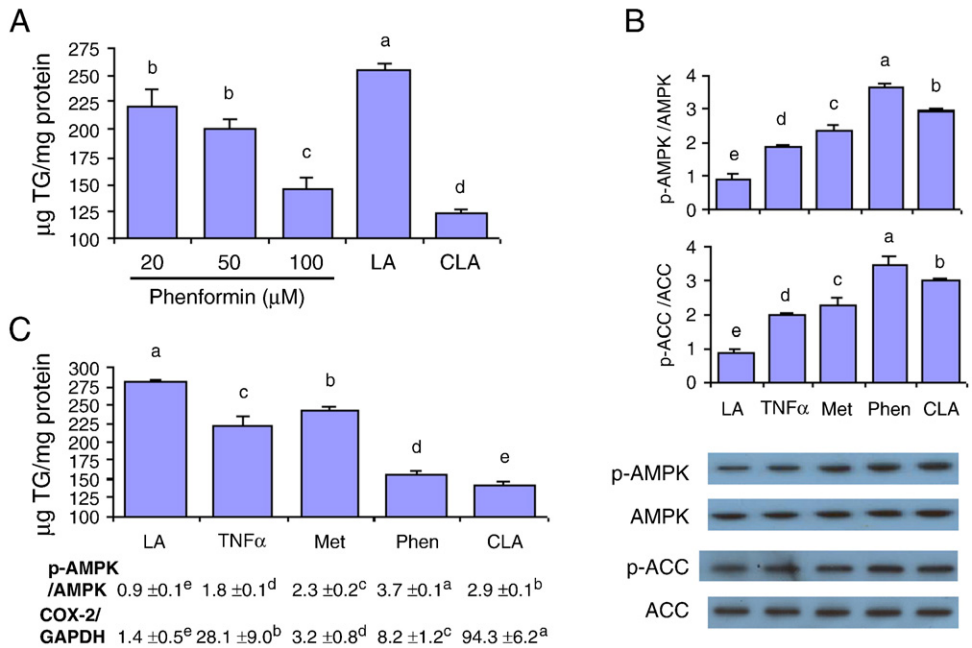

Fig. 2. Phenformin causes robust delipidation and highly activates AMPK. (A) 3T3-L1 adipocytes were treated with the indicated concentrations of phenformin, and TG levels were measured after 24 h. For comparison, the TG levels for adipocytes treated with either 100 µmol/L LA or *t*10*c*12 CLA are also shown. (B) 3T3-L1 adipocytes were treated with 100 µmol/L LA, 0.6 nmol/L TNF-α, 2 mmol/L metformin (Met), 100 µmol/L phenformin (Phen) or 100 µmol/L *t*10*c*12 CLA for 12 h. Cellular extracts were immunoblotted and the amounts of AMPK (α1 and α2), p-AMPK (α1 and α2), ACC (ACC1 and ACC2) and p-ACC (ACC1 and ACC2) were determined with specific antibodies. The immunoblots shown were representative of three independent experiments. The ratios of the phosphorylated form (p-AMPK or p-ACC) to the total amount of each respective protein (AMPK or ACC) were determined and are shown in the bar charts above the immunoblots. (C) The same treatments were used to determine their effects on TG levels after 24 h. Both the p-AMPK/AMPK ratio from (B) and the fold induction of Cox2 mRNA at 12 h, as measured by quantitative PCR, are shown below the graph. The mean±S.E.M. (n=3) of three independent experiments is shown for each type of measurement.

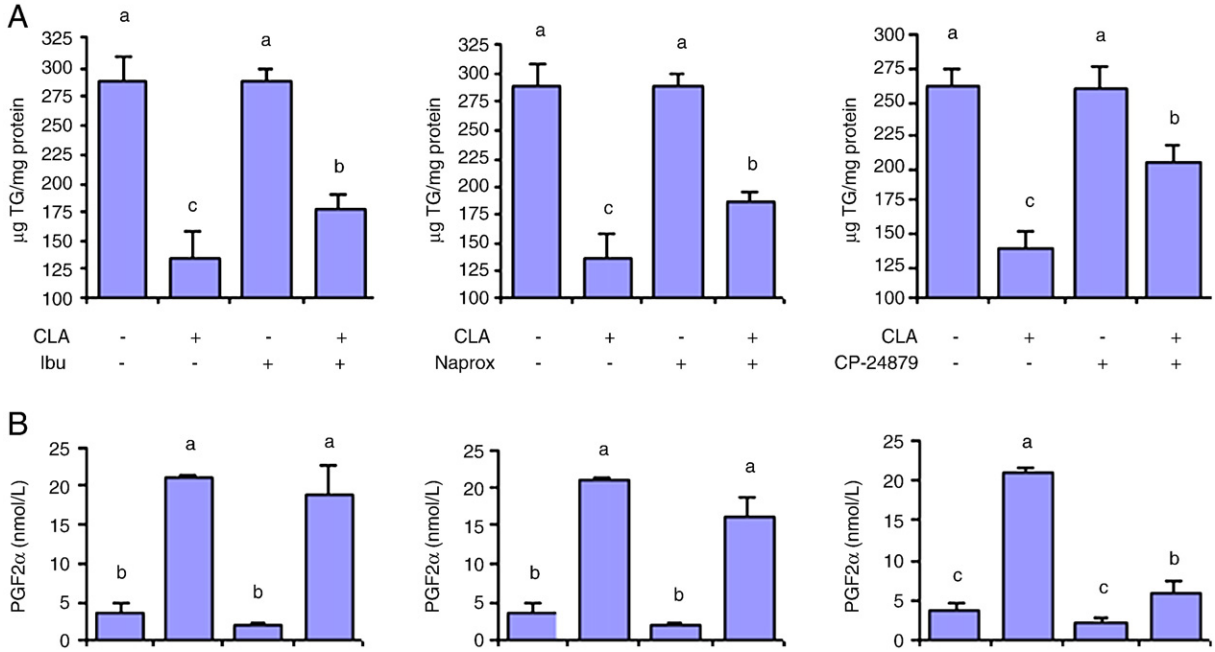

Fig. 3. Inhibitors of prostaglandin biosynthesis inhibit the reduction in TG levels and increase in PGF2α levels caused by *t*10*c*12 CLA. (A) 3T3-L1 adipocytes were treated with 100 μmol/L LA (−) or 100 μmol/L *t*10*c*12 CLA (+), with or without either 50 μmol/L ibuprofen (Ibu), 200 μmol/L naproxen (Naprox) or 50 μmol/L CP-24879, and TG levels were measured after 24 h. The interactions of *t*10*c*12 CLA with naproxen or with CP-24879 were significant, while the interaction with ibuprofen approached significance ($P \leq .08$). (B) Media from treatments of A were analyzed for PGF2α concentrations. The interaction of *t*10*c*12 CLA with ibuprofen or naproxen was not significant, but was significant for CP-24879. Each bar represents the mean±S.E.M. ($n=6$) of three independent experiments.

independently of *t*10*c*12 CLA as follows. Metformin was used to activate AMPK, as it was found previously to moderately activate AMPK without initiating a strong inflammatory response [9]. TNF-α, an inflammatory cytokine capable of causing reductions in TG levels in adipocytes [20], was used at several concentrations as the inflammatory stimulus. As a control, the biological activity of the TNF-α sample was separately verified in adipocytes by its induction of MCP-1 and CXCL1 mRNAs (Supplemental Fig. 1), which were induced to levels similar to those caused by treatments with *t*10*c*12 CLA (data not shown). Either metformin or 0.6 nmol/L TNF-α caused reductions in TG levels of 14±2% or 19±4%, respectively (Fig. 1A). The reduction in TG levels was 40±4% when both metformin and 0.6 nmol/L TNF-α were used in combination (Fig. 1A).

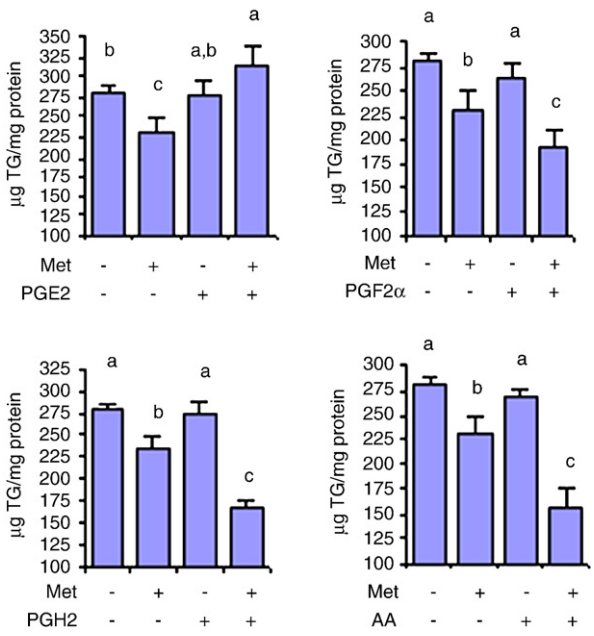

Fig. 4. Prostaglandins affect metformin's ability to reduce TG levels. 3T3-L1 adipocytes were treated with or without 2 mmol/L metformin (Met), and with or without a prostaglandin (25 μmol/L PGE_2, 25 μmol/L PGF2α or 1 μmol/L PGH2) or arachidonic acid (150 μmol/L AA). TG levels were measured after 24 h. The interaction of metformin with PGE_2, PGH2 or AA was significant, but was not significant for PGF2α. Each bar represents the mean±S.E.M. ($n=3$) of three independent experiments.

This result appeared to support our hypothesis, and to further strengthen this analysis, we checked whether the response to TNF-α was dependent on activated AMPK. Surprisingly, when the AMPK inhibitor compound C was tested for its ability to inhibit the TG loss caused by TNF-α alone, the majority of the TNF-α-mediated reduction in TG was attenuated (Fig. 1B). This latter result indicated the above experiments had more relevance for our second hypothesis that higher levels of activated AMPK were sufficient to cause robust TG loss. This hypothesis was separately tested by using phenformin, a more potent AMPK activator that is structurally related to metformin. Phenformin was used at increasing concentrations, which resulted in progressively lower TG levels (Fig. 2A). One hundred micromoles per liter of phenformin was able to reduce TG levels by 45±5%, nearly the same reduction as achieved by 100 μmol/L *t*10*c*12 CLA (50±2%; Fig. 2A).

To gain further insight into the association of the amount of activated AMPK with reduced TG levels and inflammation, the ability of TNF-α, phenformin, metformin or *t*10*c*12 CLA to activate AMPK, reduce TG levels and induce COX2 mRNA was measured. Each of these chemicals increased phosphorylation of AMPK, as well as of ACC, a known *in vivo* substrate of AMPK (Fig. 2B). Phenformin and *t*10*c*12 CLA produced more phosphorylated AMPK and ACC than TNF-α or metformin at the concentrations used for each compound (Fig. 2B). The effects of these compounds on concurrent changes in TG levels and inflammation, using COX2 mRNA as an indicator of inflammation, were also measured (Fig. 2C). The effects of these treatments suggested the amounts of activated AMPK and COX2 mRNA both

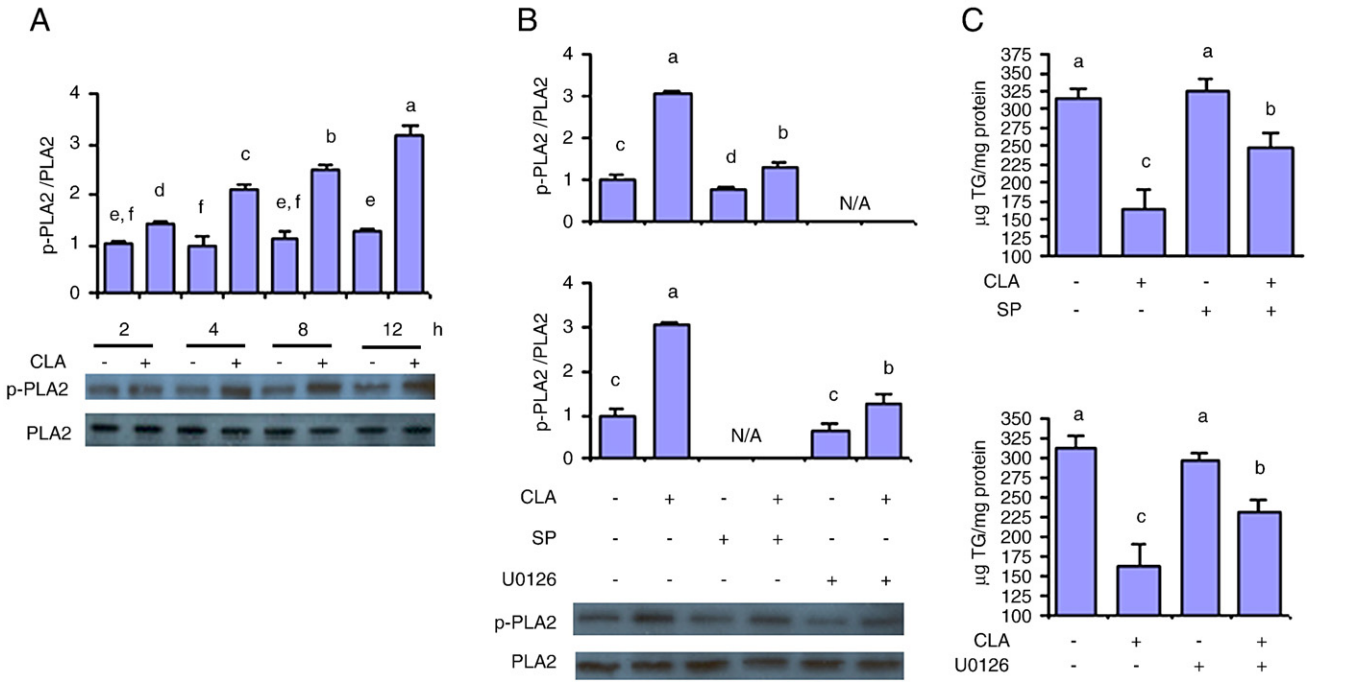

Fig. 5. *T*10*c*12 CLA induces PLA2 phosphorylation through the JNK and ERK pathways. (A) PLA2 phosphorylation was measured at various times during exposure to *t*10*c*12 CLA in 3T3-L1 adipocytes. The amount of phosphorylated PLA2 (p-PLA2) and total PLA2 was determined by immunoblots with specific antibodies. The ratio of p-PLA2 to the total PLA2 was determined and is shown in the bar chart above the immunoblots. The effects of *t*10*c*12 CLA and time were significant, but their interaction was not significant. (B) 3T3-L1 adipocytes were either not treated or pretreated for 1 h with 20 μmol/L of JNK inhibitor SP-600125 (SP) or with 20 μmol/L of MEK inhibitor U0126. Either 100 μmol/L LA or 100 μmol/L *t*10*c*12 CLA was then added for 12 h. Cellular extracts from these treatments were analyzed by immunoblot as in (A). The ratio of p-PLA2 to the total PLA2 was determined and is shown in the bar charts above the immunoblots. Both SP-600125 and U0126 had a significant inhibitory interaction with *t*10*c*12 CLA. (C) Adipocytes were treated as in (B) and TG levels were measured after 24 h. Both SP-600125 and U0126 had a significant inhibitory interaction with *t*10*c*12 CLA. The immunoblots shown in (A) and (B) are representative of three independent experiments, and each bar represents the mean±S.E.M. (n=3) of three independent experiments.

affected TG levels. *T*10*c*12 CLA or phenformin caused higher activation of AMPK and more TG loss than metformin or TNF-α. Within the *t*10*c*12 CLA and phenformin treatments, *t*10*c*12 CLA induced higher levels of COX2 mRNA and lost more TG despite its lower amount of activated AMPK. Within the metformin and TNF-α treatments, TNF-α induced higher levels of COX2 mRNA and lost more TG than metformin despite its lower amount of activated AMPK. Taken together, this data showed a trend of more highly activated AMPK causing more TG loss. For treatments with somewhat similar AMPK activation levels, higher COX2 mRNA levels seemed to be associated with more TG loss.

This latter observation supported a hypothesis that PGs might be a critical component of the inflammatory response to *t*10*c*12 CLA. The functional role of PGs was then investigated by using chemicals that inhibit PG production. Treatment of 3T3-L1 adipocytes with naproxen, ibuprofen or CP-24879 (an inhibitor of AA biosynthesis) inhibited the ability of *t*10*c*12 CLA to cause TG reductions (Fig. 3A). The effectiveness of these chemicals in reducing PG levels, using PGF2α levels as an indicator of PG production, was then determined (Fig. 3B). The modest reductions of PGF2α levels by naproxen or ibuprofen treatments were not significant, while CP-24879 caused a significant reduction in PGF2α in *t*10*c*12 CLA-treated adipocytes (Fig. 3B). The effectiveness of these inhibitors at reducing PGF2α levels was in agreement with their effectiveness at attenuating the TG loss caused by *t*10*c*12 CLA (Fig. 3A).

The functional importance of PG in the TG reductions in *t*10*c*12 CLA-treated adipocytes generated an alternative to TNFα for providing an inflammatory stimulus to test our first hypothesis (a moderate activation of AMPK and an inflammatory response are sufficient to reduce TG levels in adipocytes) independently of *t*10*c*12 CLA. This hypothesis was tested by adding a combination of metformin and a PG or AA, a precursor to PG biosynthesis, to 3T3-L1 adipocytes. Metformin alone produced a 15±4% reduction in TG levels (Fig. 4), while *t*10*c*12 CLA reduced TG levels by 47±5% (data not shown). PGE_2 alone had no effect, while the combination of PGE_2 and metformin increased the amount of TG (Fig. 4). PGF2α alone reduced TG levels by 6±2%, while PGF2α and metformin caused a 32±7% TG loss (Fig. 4). PGH2 alone had no effect on TG levels, while the combination of PGH2 and metformin reduced TG levels by 41±2% (Fig. 4). Similarly, AA alone had no effect, while AA and metformin reduced TG levels by 45±7% (Fig. 4). Metformin combined with PGH2 or AA was nearly as effective as *t*10*c*12 CLA in causing reductions of TG levels in these experiments, providing support for our first hypothesis, where the inflammatory component is supplied by exogenous PGH2 or AA.

The mechanism by which PG production is regulated in *t*10*c*12 CLA-treated adipocytes was then investigated. PLA2 releases AA by hydrolysis from the glycerol backbone of membrane lipids. PLA2 activity is activated by phosphorylation, and its phosphorylation has been reported to be increased by *t*10*c*12 CLA in human adipocytes, with the possible involvement of ERK and JNK [6]. The involvement of the ERK and JNK signaling pathways in the activation of PLA2 by phosphorylation was therefore examined. Increasing amounts of phosphorylated PLA2 were observed during longer times of exposure to *t*10*c*12 CLA (Fig. 5A). The phosphorylation of PLA2 was found to be reduced in the presence of JNK inhibitor SP600125 or MEK inhibitor U0126 (Fig. 5B), both of which also inhibited *t*10*c*12 CLA's ability to reduce TG levels (Fig. 5C).

The abilities of *t*10*c*12 CLA and phenformin to strongly activate AMPK and to cause robust reductions in TG levels suggested phenformin might initiate cellular responses very similar to those caused by *t*10*c*12 CLA. Our hypothesis was that if similar signaling

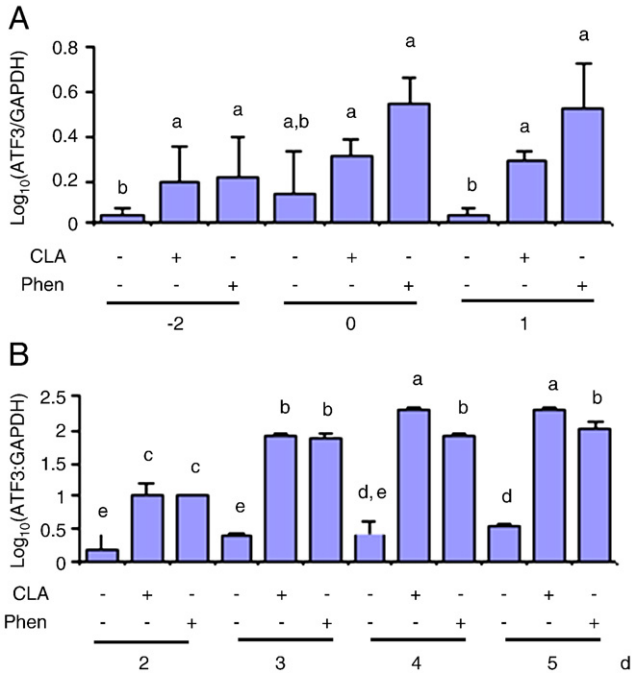

Fig. 6. The ability of phenformin or t10c12 CLA to induce the ISR is dependent on the stage of differentiation. 3T3-L1 cells at various developmental stages, either fibroblasts (2 days before confluence; −2) or differentiating adipocytes [from confluence (0) to 5 days post-confluence (Days 1–5)], were treated with 100 μmol/L LA (−), 100 μmol/L phenformin (Phen) or 100 μmol/L *t*10*c*12 CLA (CLA). RNA was isolated from the cells after 12 h of treatment and analyzed for ATF3 and GAPDH mRNA levels by reverse transcription and quantitative PCR. The relative amounts of ATF3 mRNA are shown as bar graphs. Note the change in the scale of the log10 *y*-axis starting at Day 2. The effects of the treatments and interactions were not significant for (A). For (B), the main effects of *t*10*c*12 CLA, phenformin and day of differentiation and their interaction (CLA or Phen×Day) were significant. Each bar represents the mean±S.E.M. (n=4) of four independent experiments.

pathways were being utilized by the two chemicals, the microarray results should show a high degree of similarity when analyzed for their correlation coefficients. Microarray analysis generated a list of genes with changes in transcript levels of at least fourfold in either *t*10*c*12 CLA- or phenformin-treated adipocytes (Supplementary Table 2). Previous lists of genes that responded to *t*10*c*12 CLA in the ISR, lipid metabolism or inflammatory pathways [5] were also used to compare the changes in the two treatments (Supplementary Tables 2–4). These lists were separately analyzed for the Spearman correlation coefficients and each comparison indicated a strong similarity between the transcriptional responses of 3T3-L1 adipocytes treated with phenformin or *t*10*c*12 CLA (Table 1). The changes in expression of selected functionally relevant genes were checked by real-time PCR and were in good agreement with the microarray results (Table 2).

Our earlier report indicated that differentiated adipocytes responded more robustly than their non-differentiated fibroblast precursors to *t*10*c*12 CLA, as measured by ATF3 mRNA, an indicator of the ISR [5]. The ISR is an early component of the response to *t*10*c*12 CLA and can occur in both cell types [5], unlike the TG accumulation that is specific for maturing or mature adipocytes. This differential response in differentiated adipocytes provided an additional method to compare the similarities in the responses caused by phenformin or *t*10*c*12 CLA. Additionally, phenformin is a highly polar, water-soluble small molecule, while *t*10*c*12 CLA is a hydrophobic fatty acid, providing an additional test as to whether the hydrophobic or hydrophilic nature of the chemical affected the response of cells at different differentiated states. The competence of a developmental series consisting of 3T3-L1 fibroblasts, differentiating adipocytes and mature adipocytes to respond to phenformin or *t*10*c*12 CLA was measured by the induction of ATF3 mRNA as an indicator of the ISR. The abilities of phenformin or *t*10*c*12 CLA to induce ATF3 were similar and increased dramatically at about 3 days after differentiation, with the highest levels induced at 4 and 5 days after differentiation when young and maturing adipocytes were present (Fig. 6).

The developmental competence to respond to both chemicals appeared to be the same, strengthening the premise that both chemicals activated the same developmentally regulated pathways.

4. Discussion

We examined the contributions the AMPK and inflammatory pathways, particularly PGs, make towards reducing TG levels in *t*10*c*12 CLA-treated adipocytes. A key part of our approach was to activate these pathways independently of *t*10*c*12 CLA to assess their contributions away from the complex response initiated by *t*10*c*12 CLA [4,5,19]. However, the interpretation of these "chemical mimicry" studies also relies on the fact that these pathways are activated by, and their inhibition attenuates the response to, *t*10*c*12 CLA. These chemical mimicry studies benefited from metformin's ability to moderately activate AMPK without producing a strong inflammatory response in adipocytes [9] and from phenformin's ability to strongly activate AMPK and an inflammatory response as demonstrated here. TNF-α, a pro-inflammatory cytokine, was initially used to increase the inflammatory response in adipocytes treated with metformin. TNF-α negatively regulates AMPK activity in muscle cells [32], and the extent to which TNF-α relied on AMPK activation to mediate TG reductions in adipocytes was previously unclear [20,33]. Our data indicated that TNF-α's major contribution to reducing TGs appeared to be through activated AMPK as TNF-α-treated adipocytes had increased AMPK

Table 1
Spearman correlation coefficients for gene expression changes in treatments containing t10c12 CLA or phenformin

	Correlation coefficient
All significant genes (n=541)	0.83
ISR genes (n=63)	0.87
Lipid metabolism (n=69)	0.95
Inflammation (n=49)	0.85

Each treatment contained three replicates. The number of genes being compared is represented by *n*. For the 'all significant genes,' the gene list is derived from the union of genes in either treatment that show a fourfold change in expression and a Benjamini–Hochberg-adjusted *P* value ≤.05 in at least one of the treatments being compared. The ISR, lipogenesis, and inflammation gene lists were derived from a list of genes previously found to be responsive to t10c12 CLA or tunicamycin in 3T3-L1 adipocytes [5]. The Spearman correlation was used with associated *P* values≈0.

Table 2
Fold change in mRNA levels measured by quantitative real-time PCR and microarray analysis of selected genes

Gene pathway and symbol	t10c12 CLA vs LA		Phenformin vs LA	
	RT-PCR	Microarray	RT-PCR	Microarray
ISR				
ATF3	15.1±1.9	21.9±3.1	4.8±0.6	9.6±1.8
Inflammatory				
MCP1	20.0±2.0	22.0±1.3	6.2±0.6	6.5±0.3
CXCL1	3.5±0.5	6.0±0.9	1.6±0.2	4.0±0.7
IL6	7.0±0.0	5.2±2.5	2.9±0.0	5.1±2.0
Regulatory				
C/EBPα	0.9±0.2	0.9±0.2	1.0±0.2	1.1±0.3
PPARγ	0.6±0.2	0.4±0.1	0.9±0.2	0.7±0.2
Energy related				
GLUT4	0.4±0.2	0.7±0.2	1.6±0.6	2.1±0.5
Adiponectin	1.1±0.8	1.1±0.3	1.2±0.9	1.1±0.3

activity, and the majority of TNF-α's ability to reduce TG levels was inhibited by compound C. TNF-α's activation of AMPK confounded the interpretation of its contribution to the inflammatory response, but proved useful as one of several treatments we used to establish a correlation of AMPK activity levels with TG reductions.

In addition to the level of activated AMPK, the amount of TG reduction also appeared to be influenced by the level of induction of COX2 mRNA, which supported a role for PGs in facilitating TG reductions. PG levels increase in *t*10*c*12 CLA-treated human adipocytes [6], mice [22] and 3T3-L1 adipocytes as demonstrated here. Much of the mechanism of the increased production of PGs can be explained by the activation of PLA2 [6] to increase AA production, the substrate for COX2 and the induction of COX2 mRNA levels [5,6]. Inhibitors of MEK or JNK interfered with *t*10*c*12 CLA's ability to reduce TG levels and reduced phosphorylation of PLA2, indicating the MEK and JNK pathways are involved in regulating PLA2. This is in agreement with earlier findings of the involvement of the MEK and JNK pathways in the *t*10*c*12 CLA response in human adipocytes [6,8]. Other inhibitors that interfered with either AA or PG biosynthesis also inhibited the *t*10*c*12 CLA-mediated reduction in TGs in adipocytes. These results provided support for a functional role for PGs in lowering TG levels in *t*10*c*12 CLA-treated adipocytes.

The functional role of the PGs was verified independently of *t*10*c*12 CLA by testing them in the presence of metformin. We found that metformin combined with either PGH2 or AA was nearly as effective as *t*10*c*12 CLA at reducing TG levels. Importantly, PGH2 and AA did not significantly reduce TG levels when used alone, which suggested that activated AMPK was necessary for them to enhance the TG reduction response. If we make the assumption that AMPK and PGs have similar biological activity in the *t*10*c*12 CLA-treated and alternatively treated adipocytes, then these two components account for most of the TG reduction that occurs. These results support our hypothesis that a moderate activation of AMPK and an inflammatory response are sufficient to reduce triglycerides, where most of the requirement for an inflammatory component can be provided by increased levels of PGs.

The similar effectiveness of PGH2, the precursor to the prostanoid family, and AA, the precursor to the entire eicosanoid pathway, suggests that prostanoids play the major role in the response, without ruling out minor contributions from the leukotriene component of the eicosanoid pathway. The combination of PGF2α and metformin was not quite as effective. This could be due to the participation of other eicosanoids or prostanoids derived from AA or PGH2, respectively, or possibly to a more rapid metabolic degradation of exogenous PGF2α [34]. PGE_2 in combination with metformin resulted in increased TG levels. PGE_2 can be a precursor to PGF2α, but this conversion might not be efficient with exogenously added PGE_2. Consistent with these effects, PGF2α indirectly suppresses PPARγ activity [24], while PGE_2 can also be a precursor to 15-keto-PGE_2, an agonist of PPARγ [35], which would be expected to oppose the effects of *t*10*c*12 CLA [36].

Phenformin was sufficient to cause a robust reduction in TG levels comparable to the reduction caused by *t*10*c*12 CLA, supporting our second hypothesis that strong activation of AMPK is sufficient for TG loss. This hypothesis is consistent with the first hypothesis that moderate AMPK activation and an inflammatory response are sufficient for robust TG loss, as stronger activation of AMPK is accompanied by a stronger inflammatory response as described here. We previously reported that the transcriptional responses to metformin or *t*10*c*12 CLA were also very similar, but metformin caused a weaker inflammatory response than *t*10*c*12 CLA [9]. The inability of metformin to cause the robust reductions in TG levels that result from *t*10*c*12 CLA treatments left open the question of whether *t*10*c*12 CLA activated other critical pathways or whether its stronger activation of AMPK was sufficient to explain the response to *t*10*c*12 CLA. The results with phenformin, a more potent activator of AMPK, indicate that highly activated AMPK is sufficient to cause a larger inflammatory response than metformin, although still less than that caused by *t*10*c*12 CLA which, in combination with its ability to strongly activate AMPK, can result in a robust TG reduction.

A detailed microarray analysis of the transcriptional response of the individual genes and pathways in 3T3-L1 cells and mice WAT treated with *t*10*c*12 CLA was done previously [5,17,19]. These analyses identified increases in uncoupling protein (UCP)-1 and carnitine palmitoyltransferase-1 (CPT1) transcripts, suggesting increased fatty acid oxidation could be occurring as has been observed in CLA-fed mice [37]. Fatty acid oxidation would be expected to be further enhanced by AMPK's phosphorylation of ACC, reducing the malonyl CoA pool, which typically inhibits CPT1 transport of fatty acids into mitochondria [38]. Microarray analyses also identified a large increase in many inflammatory cytokines and moderate reductions in key adipogenic transcription factors including PPARγ, CCAAT/enhancer binding protein α (C/EBPα) and sterol regulatory element binding protein (SREBP)-1c, suggesting reduced rates of lipid and cholesterol biosynthesis would result. Activated AMPK should further reduce biosynthesis by reducing the activity of key enzymes involved in the biosynthesis of fatty acids, cholesterol, glycogen and proteins [38]. Our comparison of the transcriptome response of adipocytes treated with *t*10*c*12 CLA or phenformin found that the changes in overall gene expression were very similar to those changes observed previously, particularly induction of the ISR, reduction in mRNAs of genes encoding regulatory and enzymes in the lipid biosynthesis, and induction of inflammatory pathways.

Within this overall strong similarity of the transcriptional responses, the most apparent differences were *t*10*c*12 CLA's stronger induction of the immediate early genes and of some of the inflammatory response genes. The immediate early genes are a group of genes that are predominantly transcription factors and other signaling molecules that are induced by a large variety of cellular stresses [39]. Their higher induction in *t*10*c*12 CLA-treated adipocytes indicates that *t*10*c*12 CLA either causes a more general cell stress than phenformin or, alternatively, specifically activates this pathway by an unknown mechanism. In the inflammatory response, the genes induced to higher levels in response to *t*10*c*12 CLA included COX2, suppressor of cytokine signaling 3 (SOCS3), cardiotrophin-like cytokine factor 1 (CLCF1) in the IL6 cytokine family, tumor necrosis factor receptor superfamily member 12a (TNFRSF12a) and oncostatin M receptor (OSMR). The highly induced COX2 plays an important functional role in reducing TG levels through producing PGs as demonstrated here. SOCS3 mediates local insulin resistance through inhibition of insulin receptor substrate (IRS)-1 and IRS2 [40], and can be induced by cardiotrophin [41] and, therefore, possibly by CLCF1. TNFRSF12a and OSMR are receptors for members of the TNFα or IL6 family of cytokines. Although the levels of TNF-α and IL6 mRNAs are low in 3T3-L1 adipocytes, other family members of these cytokines may signal through these receptors. Despite the overall strong similarity of the transcriptional responses to phenformin and *t*10*c*12 CLA, they were not identical, and this could be due to differences between phenformin's ability to indirectly activate AMPK by inhibition of complex 1 of the mitochondrial respiratory chain [42,43] and the still unknown pathways used by *t*10*c*12 CLA to activate AMPK.

The role of PGs *in vivo* may be less important as COX2 mRNA levels increased only two- to threefold in 24 h in WAT of mice fed *t*10*c*12 CLA [5,19]. COX2 mRNA levels then decreased to about control levels on subsequent days [19]. This is considerably less than the eightfold or 12- to 100-fold observed in human [6] and mouse adipocytes [5], respectively. PGE_2 levels were previously found to be increased about 3.4-fold in WAT of mice fed *t*10*c*12 CLA for 2 weeks. In that study, inhibition of PG production with aspirin prevented this increase in PG levels without reducing the effect of *t*10*c*12 CLA on WAT [22]. This

suggests that the role of PG in WAT might be less important *in vivo* than in adipocyte cultures. The initial and subsequent inflammatory response to *t*10*c*12 CLA treatment is much more robust and diverse in WAT than in adipocytes in culture [5], in part due to the multiple types of cells in WAT, as well as the increased infiltration of macrophages [18]. The relative contributions of these diverse inflammatory pathways to reducing WAT *in vivo* are unclear and require additional investigation.

Despite the effectiveness of *t*10*c*12 CLA, phenformin and even metformin, to a lesser extent, in reducing TG levels in mouse adipocytes and *t*10*c*12 CLA's effectiveness in mice [4] and primary human adipocytes [3], these chemicals have limited abilities to reduce adiposity in humans. The limited effectiveness of *t*10*c*12 CLA in humans remains unexplained [44], despite *t*10*c*12 CLA's preferential accumulation in adipose tissue [45]. Phenformin is no longer used to treat type 2 diabetes due to a higher frequency of lactic acidosis than occurs with metformin [46,47], and it did not reduce weight in nondiabetic patients [48]. Metformin is important in the treatment of type 2 diabetes, but it has a limited ability to either reduce weight or inhibit weight gain in patients without type 2 diabetes [49]. Our finding of a correlation of the amount of activated AMPK with the amount of TG reduction in 3T3-L1 adipocytes supports a hypothesis that higher levels of activated AMPK are needed in adipocytes for effective reduction of adiposity in humans. It is unclear how to best accomplish this, although direct AMPK activators are promising [50], and our demonstration of the differential sensitivity of adipocytes to AMPK activators increases the potential for cell selectivity in this approach. We recently demonstrated that metformin increases the ability of a moderate dose of *t*10*c*12 CLA to reduce WAT in mice [9], suggesting combinations of chemicals that activate AMPK are another possible approach. Adding a compound that leads to higher prostanoid levels in adipocytes might also be possible, but the complex biological responses to members of the prostanoid pathway [34, 51] indicate considerable research will be required to determine whether this is possible in mice and whether it can be accomplished in a therapeutically acceptable manner.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.05.005.

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