Conjugated Linoleic Acid Inhibits Differentiation of Pre- and Post-Confluent 3T3-L1 Preadipocytes But Inhibits Cell Proliferation Only in Preconfluent Cells1,2,3

Ann E. Brodie,*4 Viola A. Manning,* Kathleen R. Ferguson,* Dennis E. Jewell† and Ching Yuan Hu*

*Department of Animal Sciences, Oregon State University, Corvallis OR 97331 and †Hill’s Pet Nutrition, Inc., Topeka, KS 66601

ABSTRACT Conjugated linoleic acid (CLA; 18:2) is a group of isomers (mainly 9-cis, 11-trans and 10-trans, 12-cis) of linoleic acid. CLA is the product of rumen fermentation and can be found in the milk and muscle of ruminants. Animals fed CLA have a lower body fat content. The objective of this study was to establish the possible mechanisms by which CLA affects adipogenesis. 3T3-L1 is a well-established cell line that is used extensively in studying adipocyte biology. These cells typically grow in a culture medium until they reach confluence, at which time they are induced to differentiate by hormonal treatment (d 0). Treatment of 3T3-L1 cells with 25 to 100 μmol/L CLA inhibited differentiation in a dose-dependent manner, while linoleic acid treatment did not differ from DMSO-treated controls. Continuous treatment from d −2, −1, 0 or 2 to d 8 and from d −2 to d 0 and from d 0 to d 2 inhibited differentiation. Differentiation was monitored morphologically (oil Red-O staining), enzymatically (reduction of activity of glycerol-3-phosphate dehydrogenase), and by northern analysis of peroxisome proliferator-activated receptor γ2, CCAAT/enhancer binding protein α and adipocyte specific protein 2 mRNA. CLA inhibited cell proliferation of nonconfluent cells but did not affect cell division of confluent cells, as indicated by 5-bromo-2′-deoxyuridine incorporation and mitochondria metabolism. Therefore, CLA inhibited differentiation before confluence and during induction. However, cellular proliferation was only inhibited prior to induction. These results imply that fat reduction caused by CLA treatment may be attributed to its inhibition of both proliferation and differentiation of preadipocytes in animals.

KEY WORDS: adipocytes · conjugated linoleic acid · differentiation · proliferation · fatty acids

Conjugated linoleic acid (CLA)5 (18:2, mainly 9-cis, 11-trans and 10-trans, 12-cis), a group of isomers of linoleic acid, occurs naturally in food, with a high concentration in products from ruminant animals. CLA is effective in protecting tissues from carcinogenesis (review: Ip et al. 1994), reducing the development of atherosclerosis (Lee et al. 1994), stimulating the immune system (Cook et al. 1993) and inducing enzyme changes in mouse liver (Belury et al. 1997, Cantwell et al. 1998).

Another effect of CLA could have a profound impact on human health and animal production. Limited reports exist of CLA lowering body fat while not affecting total body mass. Pigs fed CLA deposit less subcutaneous fat, are leaner and show improved feed conversion efficiency (Dugan et al. 1997). Mice fed a CLA-supplemented diet develop lower quantities of body fat and increased carnitine palmitoyltransferase activity (Park et al. 1997, Chin et al. 1994). Mature, cultured 3T3-L1 cells treated with CLA have reduced lipoprotein lipase activity and reduced intracellular concentrations of triacylglycerol and glycerol (Park et al. 1997). Therefore, the reduction of body fat by CLA in vivo could be due to reduced fat deposition and increased lipolysis in the adipocytes.

These results prompted the natural supplement industry to market CLA to humans (e.g., Peak Nutrition, Willington CT, and PharmaNutrients, Lake Bluff, IL). The promotional literature states that by consuming CLA, body fat can be reduced. One preliminary clinical study performed by PharmaNutrients indicates that after a 90-d period, CLA consumption reduced body fat by an average of 20%. With minimal information available regarding both the mode of action of CLA in reducing animal body fat and regarding the action upon humans, more basic information is necessary to protect consumers from possible detrimental effects.

As 3T3-L1 cells are a reliable system for analyzing the development of adipocytes, we chose this system to study the
effect of CLA during preadipocyte differentiation. Although these cells are derived from mice, the basic mechanisms for fat development appear to be similar in both rodent and human cells (Mueller et al. 1998). The studies presented here show that CLA functions in at least two different ways to inhibit adipocyte development. Treatment with CLA during the cell-proliferative stage inhibited cell number and decreased 5-bromo-2′-deoxyuridine (BrdU) incorporation and subsequent differentiation. Treatment of the 3T3-L1 cells with CLA at the time of induction of differentiation (after cells reached confluence) did not affect cell numbers but did inhibit differentiation. Our results indicate the presence of two different mechanisms of action of CLA in 3T3-L1 cells. Therefore, the action of CLA with dietary supplementation may vary depending upon the stage of development of the cell, i.e., dividing cells could be severely inhibited by CLA consumption, while non-dividing cells could be less impacted.

MATERIALS AND METHODS

Growth conditions and inhibitor treatment. 3T3-L1 preadipocytes were cultured and induced to differentiate as described (Chen et al. 1997). In short, cells were placed into culture (d −6) and grown to confluence (d −2) before induction (d 0). Terminal differentiation occurred by d 12. Cells were treated with CLA, mixed isomers of 9,11, purity >99%, in dimethyl sulfoxide (DMSO) (Nu-Chek-Prep, Inc., Elysian, MN) and linoleic acid (LA, 9-cis, purity >99%, in DMSO, Nu-Chek-Prep, Inc.) at the concentrations and times indicated.

Oil Red O staining. Cells were stained with oil Red-O and hematoxylin as described by Suryawan and Hu (1993).

Northern analysis. Total RNA was extracted from cells using the guanidinium-phenol method (Chomczynski and Sacchi 1987). RNA was separated, blotted and probed with 18S as described in Brodie et al. (1986). Other blots were probed using DNA random prime labeled with digoxigenin-dUTP as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Blots were prehybridized and hybridized at 50°C and washed two times at 50°C with 1X SSPE/0.1% SDS for 15 min and one time with 0.1X SSPE/1% SDS at 60°C for 30 min. Detection was performed as recommended by the manufacturer. Source of the probe: 18S, Dr. Stephen J. Giovannoni, Oregon State University; adipose P 2 (aP2), Dr. David A. Bernhohr, University of Minnesota; CCAAT/enhancer binding protein α (C/EBPα), Dr. Stephen R. Farmer, Boston University; peroxisome proliferator-activated receptor γ2 (PPARγ2), Dr. Bruce Spiegelman, Dana Farber Cancer Institute.

Glycerol-3-phosphate dehydrogenase (GPDH) activity. The GPDH (EC 1.1.1.8) assay was performed by a spectrophotometric method for determination of the disappearance of NADH during GPDH-catalyzed reduction of dihydroxyacetone phosphate under zero-order condition (Kozak and Jensen 1974) as modified by Wise and Green (1979). The addition of CLA to the cells 1 h prior to the assay had no effect on GPDH activity. Protein was measured according to Bradford (1976).

Cell number assay. The colorimetric assay for quantitation of cell number and cell viability, based on the cleavage of the tetrazolium salt WST-1 [4-(3-[4-Iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolo]-1,3-benzene disulfonate] by mitochondrial dehydrogenases, was performed according to the manufacturer (Boehringer Mannheim #1644 807).

5-bromo-2′-deoxyuridine (BrdU) incorporation. After the indicated period of CLA treatment, cells were rinsed two times, and incorporation of BrdU was assayed after 2 h incubation, according to the kit supplied by Boehringer Mannheim (#1 647 229).

Statistical analysis. Data were analyzed using the Students t-test to compare individual treatments to the DMSO-treated control cells.

RESULTS

Adipocyte differentiation was inhibited in a dose-dependent manner by continually treating 3T3-L1 cells with CLA (25–100 μmol/L) from d −2 to d 8 (Fig. 1). LA (50 and 100 μmol/L) treatment did not cause any inhibition (data not shown). In addition to monitoring the GPDH activity, CLA-treated and untreated cells were stained with oil Red-O to visualize the fat (data not shown). After d 2, increases in the mRNA of two important adipogenic transcription factors, PPARγ2 and C/EBPα, were inhibited when CLA treatment began at d 0 (day of induction of differentiation, Fig. 2). In addition, aP2 mRNA, which is an indicator of adipocyte differentiation, also did not reach control levels (Fig. 2). However, these mRNAs were not affected when CLA treatment began at either d 2 or 4 and continued through d 7 (data not shown).

When CLA treatment was started at different times (d −2, −1, 0, and 2) and continued until d 8, GPDH enzyme activity was lower than in DMSO-treated controls. (Fig. 3). Treating 3T3-L1 cells with CLA at different intervals before addition of induction media (d −2 to 0) and during the presence of induction media (d 0 to 2) resulted in inhibition of adipocyte differentiation (Fig. 4).

Because Durgam and Fernandes (1997) showed that CLA stops cells in the G0/G1 phase of cell growth, we determined the number of cells present after CLA treatment. The tetrazolium salt WST-1 [4-(3-[4-Iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolo]-1,3-benzene disulfonate] is cleaved to formazan by enzymes that belong to the respiratory chain of the mitochondria and is active only in viable cells (see the Materials and Methods section). This assay was used to determine the number of 3T3-L1 cells remaining after treatment with CLA. We verified that WST is a reliable indicator of 3T3-L1 cell numbers by comparing the actual number of cells to the amount of formazan formed (data not shown). 3T3-L1 cells were treated with CLA, either in normal growth medium or induction medium. Induction medium was added to preconfluent cells to determine that this medium did not interfere with the WST assay. The stage of cell growth influenced the effect CLA had on cell number (Fig. 5). Regardless of the medium, CLA treatment of preconfluent cells resulted in lower cell numbers (Fig. 5A). However, confluent cells treated with CLA were unaffected (uninduced cells), or the number was greater (induced cells) (Fig. 5B).
Because CLA treatment of preconfluent cells resulted in lower cell numbers, we used BrdU incorporation to determine if DNA synthesis was inhibited. In this assay, preconfluent cells received 1 or 2 days of treatment with CLA before incubation with BrdU. BrdU incorporation was inhibited by both the 1- and 2-d CLA treatment (Fig. 6).

**DISCUSSION**

Inhibition of differentiation of 3T3-L1 cells by CLA is dose-dependent. Treatment with CLA from d 2 to 0 and d 0 to 2 inhibited differentiation as indicated by reduced GPDH activity and morphological observation. Continuous treatment with CLA after induction reduced the levels of the aP2, PPARγ2 and C/EBPα mRNA. Differentiation was inhibited by CLA treatment before and during induction of differentiation. Cell proliferation was sensitive to CLA inhibition only before confluence.

Although fatty acids stimulate adipogenesis (Amri et al. 1991), our data indicate that CLA is an exception and is inhibitory. For all variables measured, the LA-treated cells did not differ from the DMSO-treated control cells. Initially, the BrdU results indicated an inhibition of cellular proliferation by both CLA and LA, even when more cells were clearly visible in the LA-treated cells (data not shown). After several rinses to thoroughly deplete the media of the fatty acids, only the CLA was inhibitory. The presence of the fatty acid at the time of the addition of the BrdU may have inhibited BrdU uptake.
level of aP2 that is known to be regulated by PPARγ2 and C/EBPα. This indicates that CLA acts by influencing this well-described pathway. However, when cells are treated after induction (d 2), the mRNA of these three factors was not affected.

Houseknecht et al. (1998) showed that aP2 mRNA level was increased in the epididymal fat pads from diabetic fatty rats treated with CLA. Using a transfection study, they demonstrated that CLA and troglitazone can activate PPARγ transcription. They hypothesized from these data that increased PPARγ is responsible for increased aP2 mRNA. However, Okuno et al. (1998) showed in vivo that troglitazone does not increase the mRNA of PPARγ in epididymal adipose tissue. Therefore, even though Houseknecht et al. (1998) demonstrated that CLA and troglitazone increased aP2 mRNA, no data exist to support that this is a result of PPARγ transcription in vivo. Confounding the interpretation of the data by Houseknecht et al. (1998) is the fact that they assayed whole epididymal fat pads that include preadipocytes as well as mature adipocytes. Having the adipocytes at two different developmental stages leads to difficulty in interpreting their results.

In addition, fat depots are characterized by marked metabolic differences, depending upon their location (Maslowska et al. 1993). Raclot et al. (1997) showed that polyunsaturated fatty acids have different effects, depending upon the fat site. Supplementation of Ob1771 and Ob1754 preadipose cells with LA activates aP2 gene expression (Amri et al. 1991). These cells are also of epididymal origin, coming from the obese ob/ob adult mouse (Smas & Sul 1995). In another system, PPARγ2-expressing NIH 3T3 cells, LA induces expression of the aP2 mRNA, although LA is less effective than other PPAR activators (Tontonoz et al. 1994). Although we detected some stimulation by LA, the effects were not significant. Apparently fat cells from alternative sites and genetic background respond differently to fatty acids.

Animals fed CLA have less body fat (Dugan et al. 1997, Park et al. 1997). CLA reduced feed intake, improved feed conversion efficiency, decreased subcutaneous fat and increased lean deposition in pigs (Dugan et al. 1997). Park et al. (1997) concluded that the lower body fat and increased lean body mass in mice fed CLA “appear to be due in part to..."
reduced fat deposition and increased lipolysis in adipocytes, possibly coupled with enhanced fatty acid oxidation.” Our data suggest that the CLA-induced fat reduction in these animals could be attributed to its effect on adipose hyperplasia. This does not exclude the possible effect of CLA on adipocyte hypertrophy. More work is required to determine the CLA effect on lipogenesis and lipolysis in mature adipocytes.

Proliferation of MCF-7 human breast cancer cells is inhibited by CLA treatment (Durgam and Fernandes 1997). Cell cycle analysis indicated that the CLA blocked MCF-7 at the G0/G1 phase, thus reducing cell growth. Our results of CLA treatment of proliferating cells could also be explained by a block at the G0/G1 phase. As the 3T3-L1 cells would be unable to reach confluence due to CLA inhibition, there may be an incomplete response to the induction media, resulting in inhibition of differentiation.

CLA also exerts a dose-dependent reduction in proliferation of A-427, a lung adenocarcinoma cell line (Schonberg and Krokan 1995). A significant increase in lipid peroxidation is observed with the inhibition. Although the formation of malondialdehyde, the indicator of peroxidation, was completely abolished by the addition of vitamin E, growth rates were only partially restored, indicating an additional mechanism. Cantwell et al. (1998) also concluded that CLA may act as a prooxidant because they found that CLA downregulated cellular antioxidant enzymes when hepatocytes were exposed to oxygen. This oxidative action of CLA may inhibit differentiation of our 3T3-L1 cells.

Our study clearly identified an effect of CLA on proliferation and differentiation. We did not measure fat accretion, and therefore we cannot ignore the fact that CLA has affected lipogenesis or lipolysis. However, treatment of the 3T3-L1 cells was unable to reach confluence due to CLA inhibition, there may be incomplete response to the induction media, resulting in inhibition of differentiation.

ACKNOWLEDGMENTS

We appreciate the supply of probes from Steve Giovannoni, David Bernhohr, Stephen Farmer and Bruce Spiegelman.

LITERATURE CITED


