Conjugated Linoleic Acid Reduces Phorbol Ester-Induced Prostaglandin $F_{2\alpha}$ Production by Bovine Endometrial Cells¹

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ABSTRACT

Recent interest in conjugated linoleic acid (CLA) research stems from the well-documented anticarcinogenic, antiatherogenic, antidiabetic, and antiobesity properties of CLA in animal models. The objective of this study was to examine the effects of 2 CLA isomers (*cis*-9,*trans*-11 and *trans*-10,*cis*-12) on phorbol 12,13-dibutyrate (PDBu)-induced $PGF_{2\alpha}$ production in cultured bovine endometrial (BEND) cells. Confluent BEND cells were incubated in the absence (control) or presence of 100 μM each of linoleic acid, *cis*-9,*trans*-11 CLA, or *trans*-10,*cis*-12 CLA for 24 h. After incubation, cells were rinsed and then stimulated with PDBu (100 ng/mL) for 6 h. Compared with untreated cells, PDBu stimulated $PGF_{2\alpha}$ secretion (+25-fold) within 6 h. The increases in $PGF_{2\alpha}$ secretion were paralleled by significant induction of prostaglandin endoperoxide synthase-2 (PGHS-2) mRNA (+63-fold) and protein (+1.6-fold) expression. In spite of stimulatory effects on PGHS-2 and peroxisome proliferator-activated receptor δ (PPAR δ) mRNA responses, CLA greatly decreased $PGF_{2\alpha}$ production by PDBu-stimulated BEND cells. There was no evidence for PDBu or CLA modulation of PPAR δ protein synthesis in cultured BEND cells. Results indicated that CLA modulation of $PGF_{2\alpha}$ production by BEND cells was not mediated through PGHS-2 or PPAR δ gene repression.

Key words: conjugated linoleic acid, uterus, prostaglandin $F_{2\alpha}$, cow

INTRODUCTION

Prostaglandin endoperoxide synthase (PGHS) is a rate-limiting enzyme that catalyzes the initial step of prostaglandin (PG) biosynthesis from arachidonic acid (Dubois et al., 1993; Herschman, 1996). This enzyme is encoded by 2 separate genes, PGHS-1 and PGHS-2, both of which participate in the formation of a variety

of eicosanoids, including PGD_2 , PGE_2 , $PGF_{2\alpha}$, PGI_2 , and thromboxane A. Prostaglandin endoperoxide synthase-1 is expressed constitutively, whereas PGHS-2 is an inducible early gene that is upregulated by various stimuli, including cytokines, growth factors, and tumor promoters (Kujubu et al., 1991; Dubois et al., 1994; Arslan and Zingg, 1996). Prostaglandin endoperoxide synthase-2 is readily expressed in bovine endometrial (BEND) cells and is thought to mediate phorbol 12,13-dibutyrate (PDBu)-induced PG synthesis in endometrial cells (Binelli et al., 2000; Badinga et al., 2002; Mattos et al., 2003). The BEND cells possess characteristics typical of uterine epithelial cells, with a cobblestone appearance and expression of cytokeratin, and therefore constitute an excellent model for examining dietary regulation of $PGF_{2\alpha}$ production in reproductive tissues.

Conjugated linoleic acid (CLA) is a collective term describing a mixture of positional and geometric dienoic isomers of linoleic acid (LA; 18:2). To date, up to 17 CLA isomers have been described, with double bonds ranging in position from carbons 6 and 8 to carbons 12 and 14 (Dhiman et al., 2005). Unlike naturally occurring unsaturated fatty acids, the double bonds in CLA occur on adjacent carbons and are not separated by a methylene group. Essentially all *cis*- and *trans*-isomeric conformations of CLA have been identified in food. However, the predominant CLA in ruminant fats is the *cis*-9,*trans*-11 (**c9,t11**) isomer, which accounts for more than 80% of total CLA isomers in dairy products and 75% of those in beef fats (Chin et al., 1992). This isomer originates from CLA produced by rumen bacteria as an intermediate in the biohydrogenation of LA or from tissue synthesis of CLA by Δ^9 -desaturase conversion of *trans*-11 18:1 fatty acid (vaccenic acid; Dhiman et al., 2005). Although available evidence indicates that CLA attenuates PG production in mammalian species (Harris et al., 2001; Urquhart et al., 2002), the mechanism by which CLA alters endometrial PG biosynthesis is not fully understood.

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that regulate multiple physiological processes, including inflammation, development, and lipid metabolism (Escher and

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Wahli, 2000). Tissue distribution of PPAR α and PPAR γ gene transcripts indicates that they play roles in fatty acid catabolism and adipogenesis, respectively (Braissant et al., 1996). Much less is known about the function and regulation of PPAR δ , although it is highly expressed in the brain, colon, and skin (Braissant et al., 1996). We recently detected an inverse relationship between PPAR δ and uterine expression of bovine estrogen receptor α and PGHS-2 genes (Balaguer et al., 2005), suggesting that this nuclear receptor subtype may play an important role in the control of reproductive processes in domestic mammals.

Given the lack of information relating uterine components of the PG cascade and supplemental CLA, the objectives of this study were 1) to examine the effects of 2 CLA isomers on PDBu-induced PGF $_{2\alpha}$ production and 2) to determine the relationships between supplemental CLA and endometrial expression of PGHS-2 and PPAR δ genes.

MATERIALS AND METHODS

Materials

Polystyrene tissue culture dishes (100 $\times$ 20 mm) were purchased from Corning (Corning Glass Works, Corning, NY). The Hams F-12 medium, antibiotic/antimycotic, PDBu, horse serum, D-valine, insulin, fatty acid-free BSA, aprotinin, leupeptin, and pepstatin were from Sigma Chemical Co. (St. Louis, MO). The minimum essential medium and fetal bovine serum were from US Biologicals (Swampscott, MA) and Atlanta Biologicals (Norcross, GA), respectively. Linoleic acid, *c9,t11* CLA, *trans*-10,*cis*-12 (**t10,c12**) CLA, PGHS-2 antibody, and PGF $_{2\alpha}$ standard were from Cayman Chemicals (Ann Arbor, MI). The PPAR δ and secondary antirabbit IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence kit was purchased from PerkinElmer (Boston, MA). Hanks' balanced salt solution (**HBSS**) and TriZol reagent were from Gibco BRL (Carlsbad, CA). Isotopically-labeled PGF $_{2\alpha}$ (5,6,8,9,11,12,14,15[n - 3 H]PGF $_{2\alpha}$; 208 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ). The anti-PGF $_{2\alpha}$ antibody was purchased from Oxford Biomedicals (Oxford, MI). BioTrans nylon membrane and [α - 32 P]deoxycytidine triphosphate (SA 3,000 Ci/nmol) were from MP Biomedicals (Atlanta, GA). The PGHS-2 cDNA probe was cloned from an ovarian follicular cDNA library (Liu and Belury, 1998), whereas the PPAR δ probe was generated from bovine endometrial RNA (Balaguer et al., 2005).

Cell Culture and Treatment

Immortalized BEND cells (American Type Culture Collection # CRL-2398, Manassas, VA) were suspended

Figure 1. Experimental manipulations to study the effects of conjugated linoleic acid (CLA) isomers on PGF $_{2\alpha}$ synthesis in cultured bovine endometrial cells. BEND = bovine endometrial; PDBu = phorbol 12,13-dibutyrate; LA = linoleic acid; *c9,t11* = *cis*-9,*trans*-11 CLA; *t10,c12* = *trans*-10,*cis*-12 CLA; RIA = radioimmunoassay; PGHS-2 = prostaglandin endoperoxide synthase-2; PPAR δ = peroxisome proliferator-activated receptor δ .

(0.5×10^6 cells/mL) in growth medium (40% Hams F-12, 40% minimum essential medium, 1% antibiotic/antimycotic, 200 U/L of insulin, 0.343 g/L of D-valine, 10% heat-inactivated fetal bovine serum, and 10% horse serum) and incubated at 37°C in a 95% air–5% CO $_2$ environment. Each culture was replenished with fresh medium on the second and fourth day after seeding, and cells reached confluence after 4 to 5 d of culture.

To examine the effects of supplemental CLA on uterine PGF $_{2\alpha}$ production, BEND cells were treated with PDBu alone (100 ng/mL) or PDBu in combination with 100 μ M of LA, *c9,t11* CLA, or *t10,c12* CLA (Figure 1). Fatty acids were complexed with BSA (1:3 ratio) for 2 h, and then treatments were applied to cells for a 24-h period. After the 24-h exposure to fatty acids, cells were rinsed with HBSS and challenged with PDBu for another 6 h. Cell-conditioned media were collected and stored at –20°C for subsequent PGF $_{2\alpha}$ radioimmunoassay. The remaining cell monolayer was rinsed with HBSS, lysed in TriZol reagent, and stored at –80°C for subsequent mRNA analysis.

PGF $_{2\alpha}$ Radioimmunoassay

The PGF $_{2\alpha}$ concentration in cell-conditioned media was measured in duplicate as described by Binelli et al. (2000). Assay sensitivity was 0.1 ng/mL and intra- and interassay coefficients of variation were 8.2 and

15.8%, respectively. Final $\text{PGF}_{2\alpha}$ concentrations were expressed as picograms per milliliter of media because the total cell numbers did not vary among Petri dishes (Caldari-Torres et al., 2006).

RNA Isolation and Analysis

Total cellular RNA was isolated from control and treated BEND cell cultures using TriZol reagent according to the manufacturer's instructions. Ten micrograms of RNA was fractionated in 1.0% agarose-formaldehyde gel and blotted to a BioTrans nylon membrane by capillary action. The RNA was cross-linked to the membrane by UV irradiation and baked at 80°C for 1 h. The RNA filters were hybridized consecutively with random primer-labeled PGHS-2 and PPAR δ cDNA probes. The PGHS-2 cDNA probe was a gift from Jean Sirois (Université de Montreal, St-Hyacinthe, Canada), whereas the cDNA probe for PPAR δ was generated using a set of primers (forward: 5'-CACTCTCACTGCTG GACAA-3'; reverse: 5'-TGCGGTTCTTCTTCTGGATT-3') designed from the bovine gene sequence (GenBank accession number: AF 229357). The size (216 bp) and identity of the PCR product were further verified by DNA sequencing before its use in Northern blot analyses. After hybridization, RNA filters were washed for 20 min in 50 mL of 2× saline sodium citrate–0.1% SDS at room temperature, followed by two 15-min washes in 0.1× saline sodium citrate–0.1% SDS at 42°C. The filters were blotted dry and exposed to X-ray films for 6 to 24 h at –80°C. Hybridization signals for each target gene were quantified by densitometric analysis and are reported as ratios of target gene values over the corresponding 18S rRNA values.

Western Blot Analysis of PGHS-2 and PPAR δ

For Western blot analyses, whole cell lysates were prepared as described by Binelli et al. (2000). Briefly, 500 μL of ice-cold whole cell extract buffer (50 mM Tris, pH 8.0; 300 mM NaCl; 20 mM NaF; 1 mM Na_3VO_4 ; 1 mM $\text{Na}_4\text{P}_2\text{O}_7$; 1 mM EDTA; 1 mM ethylene glycol-bis 2 aminoethylether-*N,N,N',N'*-tetraacetic acid; 1 mM dithiothreitol; 0.5 mM phenylmethanesulfonyl fluoride; 10% vol/vol glycerol, 0.5% vol/vol Nonidet P-40, and 10 $\mu\text{g}/\text{mL}$ each of aprotinin, leupeptin, and pepstatin) were added to each dish and cells were collected in 1.5-mL microcentrifuge tubes and incubated for 30 min at 4°C. Cell lysates were centrifuged at 4°C for 2 min (13,000 $\times g$) to remove cell debris. Protein concentration in the supernatant was determined by the method of Lowry et al. (1951). Protein (25 μg) from each dish was resolved on a 7.5% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. The membrane

was blocked for 2 h in 5% (wt/vol) nonfat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (pH 7.4), rinsed with TBS containing 0.1% Tween-20, and hybridized with antibodies against either PGHS-2 or PPAR δ diluted (1:500) in TBS containing 5% nonfat dried milk. Target proteins were detected by enhanced chemiluminescence and the relative abundance of each protein was quantified by densitometric analysis (Kodak EDAS 290; Kodak, Rochester, NY).

Statistical Analyses

Prostaglandin $\text{F}_{2\alpha}$, mRNA, and protein responses were analyzed using the GLM procedure in the SAS software package (SAS Institute Inc., Cary, NC). For $\text{PGF}_{2\alpha}$ concentration, the sources of variation included experiment, treatment, experiment $\times$ treatment interaction, and dish (experiment $\times$ treatment). The dish nested within experiment and treatment was considered a random variable; therefore, the dish variance was used as an error term to test the effects of experiment, treatment, and experiment $\times$ treatment interaction. For PGHS-2 and PPAR δ mRNA responses, the statistical models included experiment, treatment, and experiment $\times$ treatment interaction. Densitometric values for each target gene were expressed as ratios over the values for 18S ribosomal RNA. For Western blot analysis, the statistical model included only the main effect of treatment. For all responses, when treatment effects were detected ($P < 0.05$), means were separated using preplanned orthogonal contrasts.

RESULTS

Stimulation of BEND cells with PDBu increased ($P < 0.01$) $\text{PGF}_{2\alpha}$ production by 23-fold (Figure 2A). The increase in $\text{PGF}_{2\alpha}$ production coincided with marked induction ($P < 0.01$) of PGHS-2 mRNA (Figure 2B) and PGHS-2 protein (Figure 2C) expression. Conversely, treatment with PDBu had no detectable effect on PPAR δ mRNA (Figure 3A) or PPAR δ protein (Figure 3B) concentrations in cultured BEND cells.

Preincubation of BEND cells with CLA, and not LA, reduced ($P < 0.01$) the $\text{PGF}_{2\alpha}$ response to PDBu by 24% (Figure 4). The 2 CLA isomers were equally potent in decreasing the $\text{PGF}_{2\alpha}$ response to PDBu (Figure 4). The concentration of PGHS-2 mRNA transcript was greater ($P < 0.01$) in BEND cells cotreated with PDBu and the 3 fatty acids than in the cells treated with PDBu alone (Figure 5A). The fatty acid treatment had a negligible effect on PGHS-2 protein response to PDBu (Figure 5B). The relative abundance of PPAR δ mRNA transcript was greater ($P < 0.01$) in BEND cells coincubated with PDBu and fatty acids than in those treated with PDBu alone

Figure 2. Effect of phorbol 12,13-dibutyrate (PDBu) on PGF_{2α} (A), prostaglandin endoperoxide synthase-2 (PGHS-2) mRNA (B), and PGHS-2 protein (C) concentrations in cultured bovine endometrial cells. For Northern and Western blot analyses, the top panel shows a representative Northern or Western blot, whereas the bottom panel represents means ± SEM calculated over 2 experiments (n = 4 for each treatment). Asterisks above histograms indicate differences between treatments ($P < 0.01$).

(Figure 6A). None of the fatty acids studied had detectable effects on the PPAR δ protein concentration in PDBu-stimulated BEND cells (Figure 6B).

DISCUSSION

Conjugated linoleic acid is a collective term describing a mixture of positional and geometric isomers of

Figure 3. Peroxisome proliferator-activated receptor δ (PPAR δ) mRNA (A) and PPAR δ protein (B) concentrations in bovine endometrial cells incubated in the absence (control) or presence of phorbol 12,13-dibutyrate (PDBu). For each response, the top panel shows a representative Northern or Western blot, whereas the bottom panel represents means ± SEM calculated over 2 experiments (n = 4 for each treatment).

LA. They occur naturally in ruminant fats and dairy products and have been shown to reduce the incidence of tumors, activate the immune function, reduce body fat deposition, and increase feed efficiency and growth in animal models (Pariza et al., 2001). Results of this study extend previous observations that supplemental CLA reduces eicosanoid production in mammalian models (Whigham et al., 2002; Ogborn et al., 2003; Cheng et al., 2003). The effect was CLA-specific because LA, the parent molecule of CLA, had no detectable effect on PGF_{2α} response to PDBu.

Figure 4. Effect of linoleic acid (LA) and conjugated linoleic acid (CLA) isomers [*cis*-9,*trans*-11 (*c9,t11*) and *trans*-10,*cis*-12 (*t10,c12*)] on PGF_{2α} response to phorbol 12,13-dibutyrate (PDBu) in cultured bovine endometrial cells. Treatment means were compared using preplanned orthogonal contrasts. Contrast 1: PDBu vs. (LA + *c9,t11* + *t10,c12*), $P < 0.01$. Contrast 2: LA vs. CLA (*c9,t11* + *t10,c12*), $P < 0.01$. Contrast 3: *c9,t11* CLA vs. *t10,c12* CLA, $P = 0.4$.

The mechanism underlying CLA inhibition of PG production is not fully understood and may involve more than one pathway. Banni et al. (1999) reported that CLA supplementation of rat mammary tissue increased the accumulation of CLA metabolites at the expense of LA metabolites in the tissue. Because CLA metabolites are potent inhibitors of PGHS-2 and lipoxygenase enzymes (Nugteren and Christ-Hazelhof, 1987), it has been suggested that supplemental CLA may reduce PG production, in part by inhibiting PGHS-2 enzyme activity in cultured mammalian cells. In addition, polyunsaturated fatty acids do not alter phospholipase A₂ protein levels in BEND cells (Mattos et al., 2003), suggesting that CLA may compete with arachidonic acid for interaction with PGHS-2. Other studies have indicated that supplemental CLA may compete with LA in elongation and desaturation steps in the synthesis of arachidonic acid, the major precursor of PGE₂ and PGF_{2α} (Thompson et al., 1997; Liu and Belury, 1998). In support of this hypothesis, incubation of murine keratinocytes with CLA reduced the content of arachidonic acid in cells, whereas LA increased the arachidonic acid content. The decreased arachidonic acid content in CLA-treated cells was associated with a marked decrease in PGE₂ production, whereas LA enhanced PGE₂ production (Liu and Belury, 1998).

In addition to decreasing the arachidonic acid content in the cell, CLA may have effects at the level of gene expression. Consistent with this hypothesis, Iwakiri et al. (2002) showed that CLA supplementation greatly

Figure 5. Effects of linoleic acid (LA) and conjugated linoleic acid (CLA) on prostaglandin endoperoxide synthase-2 (PGHS-2) mRNA (A) and PGHS-2 protein (B) responses to phorbol 12,13-dibutyrate (PDBu) in bovine endometrial cells. For each response, the top panel shows a representative Northern or Western blot, whereas the bottom panel represents means $\pm$ SEM calculated over 2 experiments ($n = 4$ for each treatment). Treatment means were compared using preplanned orthogonal contrasts. For PGHS-2 mRNA, contrast 1: PDBu vs. [LA + *cis*-9,*trans*-11 CLA (*c9,t11*) + *trans*-10,*cis*-12 CLA (*t10,c12*)], $P < 0.01$. Contrast 2: LA vs. CLA (*c9,t11* + *t10,c12*), $P < 0.01$. Contrast 3: *c9,t11* CLA vs. *t10,c12* CLA, $P < 0.01$. For PGHS-2 protein, contrast 1: PDBu vs. (LA + *c9,t11* CLA + *t10,c12* CLA), $P = 0.2$. Contrast 2: LA vs. CLA (*c9,t11* + *t10,c12*), $P = 0.2$. Contrast 3: *c9,t11* CLA vs. *t10,c12* CLA, $P = 0.3$.

reduced mRNA abundance of PGHS-2 and inducible nitric oxide synthase in activated macrophages. The authors postulated that CLA may inhibit cellular PGHS-2 gene expression through inactivation of nuclear factor $\kappa\beta$. It is unlikely that this mechanism of gene repression is functional in BEND cells, because

Figure 6. Effects of linoleic acid (LA) and conjugated linoleic acid (CLA) on peroxisome proliferator-activated receptor δ (PPAR δ) mRNA (A) and PPAR δ protein (B) responses to phorbol 12,13-dibutyrate (PDBu) in bovine endometrial cells. For each response, the top panel shows a representative Northern or Western blot, whereas the bottom panel represents means $\pm$ SEM calculated over 2 experiments ($n = 4$ for each treatment). Treatment means were compared using pre-planned orthogonal contrasts. For PPAR δ mRNA, contrast 1: PDBu vs. [LA + *cis*-9, *trans*-11 CLA (*c9,t11*) + *trans*-10, *cis*-12 CLA (*t10,c12*)], $P < 0.01$. Contrast 2: LA vs. CLA (*c9,t11* + *t10,c12*), $P < 0.05$. Contrast 3: *c9,t11* CLA vs. *t10,c12* CLA, $P < 0.01$. For PPAR δ protein, PDBu vs. (LA + *c9,t11* CLA + *t10,c12* CLA), $P = 0.4$. Contrast 2: LA vs. CLA (*c9,t11* + *t10,c12*), $P = 0.2$. Contrast 3: *c9,t11* CLA vs. *t10,c12* CLA, $P = 0.2$.

CLA failed to attenuate PGHS-2 mRNA response in PDBu-stimulated BEND cells. The observation that CLA up-regulated PGHS-2 gene expression in BEND cells is consistent with a previous report (Nakanishi et al., 2003), and suggests that, in this cell type, CLA modulation of PG production may involve posttran-

scriptional modification of PGHS-2 activity. This hypothesis does not rule out the possibility that the substrate availability for PG biosynthesis may also be limiting in CLA-treated BEND cells.

Our laboratory recently reported an inverse relationship between PPAR δ and estrogen receptor α and PGHS-2 mRNA concentrations in bovine endometrial tissues collected from d 3 and 7 cyclic Holstein cows (Balaguer et al., 2005). Therefore, we sought to examine whether CLA-induced attenuation of PGF_{2 α} production by BEND cells may be associated with altered PPAR δ mRNA or protein synthesis. Priming of BEND cells with *c9,t11* and *t10,c12* CLA isomers enhanced PPAR δ mRNA response to PDBu, but had no detectable effect on the PPAR δ protein concentration. There was no apparent relationship between PPAR δ mRNA content and PGF_{2 α} concentration in control or PDBu-treated cells. These findings suggest that PPAR δ is probably not the major mediating factor in CLA-mediated reduction of uterine eicosanoid production in cattle.

CONCLUSIONS

Priming of BEND cells with *c9,t11* and *t10,c12* CLA isomers greatly decreased the PGF_{2 α} response to PDBu. This inhibitory effect was CLA specific, because LA, the parent molecule of CLA, had a negligible effect on the endometrial PGF_{2 α} response to PDBu. Coincubation with both CLA isomers enhanced PGHS-2 and PPAR mRNA responses to PDBu, suggesting that these fatty acids alter PGF_{2 α} production through a mechanism that does not involve PGHS-2 or PPAR δ gene repression. Further studies are needed to test whether CLA isomers affect the activity of various enzymes and transcription factors involved in the PG biosynthetic cascade.

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