Differential Effects of n-3 and n-6 Fatty Acids on Prostaglandin $F_{2\alpha}$ Production by Bovine Endometrial Cells¹

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ABSTRACT

Recent studies have implicated n-3 polyunsaturated fatty acids in the reduction of eicosanoid production in the bovine uterus. The objective of this study was to determine whether the effect of eicosapentaenoic acid (EPA; $C_{20:5}$, n-3) on $PGF_{2\alpha}$ production by bovine endometrial (BEND) cells is influenced by the quantity of linoleic acid ($C_{18:2}$, n-6) in the incubation medium. Confluent BEND cells were incubated in the absence (control) or presence of 100 μM of EPA for 24 h. After incubation, cells were rinsed and then stimulated with phorbol 12,13-dibutyrate (PDBu; 100 ng/mL) for 6 h. Additional sets of culture dishes were treated with a combination of EPA and increasing n-6/n-3 fatty acid ratios for 24 h and then challenged with PDBu for 6 h. The PDBu stimulated $PGF_{2\alpha}$ secretion and upregulated steady-state concentrations of prostaglandin endoperoxide synthase-2 and peroxisome proliferator-activated receptor delta mRNA within 6 h. Preincubation of BEND cells with EPA for 24 h decreased $PGF_{2\alpha}$ response to phorbol ester, but had no detectable effects on prostaglandin endoperoxide synthase-2 or peroxisome proliferator-activated receptor delta mRNA abundance in PDBu-stimulated BEND cells. The inhibitory effect of EPA on $PGF_{2\alpha}$ production was reverted in BEND cells treated with an increasing n-6-to-n-3 fatty acid ratio. Findings indicate that the net inhibition of endometrial $PGF_{2\alpha}$ bioynthesis by n-3 fatty acids may vary depending on the ratio of n-6 to n-3 fatty acids in the uterus.

Key words: fatty acid, uterus, prostaglandin $F_{2\alpha}$, cattle

INTRODUCTION

In ruminants, pregnancy establishment and embryo survival depend on attenuation of episodic release of $PGF_{2\alpha}$ from the endometrium (McCracken et al., 1972; Thatcher et al., 1984). The rate-limiting step in prosta-

glandin (PG) synthesis is the cleavage of sn-2 fatty acyl ester bond of membrane phospholipids by cytosolic phospholipase A_2 (Van den Bosch, 1980; Irvine, 1982). The arachidonic acid (ARA) that is released by phospholipid hydrolysis is acted on by PG endoperoxide synthase (PGHS) to form PGH_2 , which then is converted to $PGF_{2\alpha}$.

Recent studies have implicated n-3 polyunsaturated fatty acids in the reduction of eicosanoid synthesis in the bovine uterus (Mattos et al., 2002; Petit et al., 2002; Mattos et al., 2004). For example, infusing ewes with 3 mL/kg of BW per day of an emulsion of fish oil containing 30% eicosapentaenoic acid (EPA) and 20% docosahexaenoic acid (DHA) blocked a betamethasone-induced increase in plasma concentration of $PGF_{2\alpha}$ and delayed the occurrence of parturition (Baguma-Nibasheka et al., 1999). In humans, consumption of large quantities of fish oil resulted in delayed parturition, conceivably because of reduced secretion of $PGF_{2\alpha}$ (Olsen et al., 1992). Mattos et al. (2002) reported that supplementing the diet of lactating dairy cows with fish meal (rich in n-3 fatty acids) tended to decrease plasma 13,14 dihydro-15 ketoprostaglandin concentrations after an oxytocin injection. The same researchers (Mattos et al., 2003) later showed that EPA and DHA were potent inhibitors of $PGF_{2\alpha}$ production, whereas linoleic acid (LA) had no effect on $PGF_{2\alpha}$ response to phorbol 12,13 dibutyrate (PDBu) in cultured bovine endometrial (BEND) cells. It has been postulated that supplemental fatty acids may inhibit $PGF_{2\alpha}$ secretion by decreasing the availability of the precursor ARA, increasing the concentration of fatty acids that compete with ARA for processing by PGHS, or inhibiting PGHS synthesis, activity, or both (Mattos et al., 2000; Cheng et al., 2001).

Polyunsaturated fatty acids elicit several metabolic changes through alteration of the activity or synthesis of nuclear peroxisome proliferator-activated receptors (PPAR; Price et al., 2000). There are 3 subtypes of PPAR ($PPAR\alpha$, δ/β , and γ), all of which appear to have distinct patterns of expression and functional roles (Braissant and Wahli, 1998). Subtype $PPAR\alpha$ is involved primarily in the control of lipid catabolism (Jalouli et al., 2003; Lee et al., 2003; Schiffrin et al., 2003),

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whereas subtype PPAR γ regulates adipocyte differentiation, lipid storage, and insulin sensitivity (Chawla et al., 2003; Schiffrin et al., 2003). Much less is known about the function of PPAR δ , although it is highly expressed in the brain, colon, and skin (Mano et al., 2000; Matsuura et al., 1999). We recently detected an inverse relationship between PPAR δ and uterine expression of estrogen receptor alpha and PGHS-2 genes (Balaguer et al., 2005), suggesting that this nuclear receptor may play an important role in the control of reproductive processes in mammalian species.

Conventional cattle diets contain a mixture of n-3 and n-6 fatty acids. However, there is currently no information as to how these fatty acids may interact to alter uterine endometrial PGF $_{2\alpha}$ production in cattle. Objectives of this study were 1) to examine the effect of EPA on PGF $_{2\alpha}$ production in cultured BEND cells and 2) to determine whether the effect of EPA on PGF $_{2\alpha}$ production is influenced by the ratio of n-6 to n-3 fatty acids in the incubation medium.

MATERIALS AND METHODS

Materials

Polystyrene tissue culture dishes (100 $\times$ 20 mm) were purchased from Corning (Corning Glass Works, Corning, NY). The Ham F-12 medium, antibiotic antimycotic, PDBu, horse serum, D-valine, insulin, and fatty acid-free BSA were from Sigma Chemical Co. (St. Louis, MO). The Minimum Essential Medium and fetal bovine serum were from U.S. Biologicals (Swampscott, MA) and Atlanta Biologicals (Norcross, GA), respectively. Eicosapentaenoic acid, LA, and PGF $_{2\alpha}$ standard were from Cayman Chemicals (Ann Arbor, MI). 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 antiPGF $_{2\alpha}$ antibody was purchased from Oxford Biomedicals (Oxford, MI). BioTrans nylon membrane and [α - 32 P]deoxycytidine triphosphate (SA 3000 Ci/nmol) were from MP Biomedicals (Atlanta, GA). The PGHS-2 cDNA probe was cloned from an ovarian follicular cDNA library (Liu et al., 1999).

Cell Culture and Treatment

Immortalized BEND cells (American Type Culture Collection #CRL-2398, Manassas, VA) were cultured as described by Mattos et al. (2003) with the following modifications. Cells were suspended (0.5×10^6 cells/mL) in growth medium (40% Ham 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 every 2 d until cells reached confluence.

To examine the effect of EPA on PDBu-stimulated PGF $_{2\alpha}$ secretion, confluent BEND cells were rinsed twice with HBSS and incubated in serum-free medium with or without EPA (100 μ M) for 24 h (Figure 1A). The fatty acid was complexed with BSA at a molar ratio of 2:1 before being added to the cultures. The 24-h incubation period with fatty acid was chosen based on reported effects of EPA and DHA on phorbol ester-induced PGF $_{2\alpha}$ secretion by BEND cells after 24 h (Mattos et al., 2003). Medium was then removed, and the cells were incubated in serum-free medium with or without PDBu (100 ng/mL) for an additional 6 h (Figure 1A). After PDBu challenge, samples (0.5 mL) of cell-conditioned media were collected and stored at -20°C until assayed for PGF $_{2\alpha}$ concentration. The remaining cell monolayers were rinsed in ice-cold HBSS, lysed with TriZol, and stored at -80°C until PGHS-2 and PPAR δ mRNA quantifications.

To determine whether the effect of EPA on endometrial PGF $_{2\alpha}$ secretion is influenced by the ratio of n-6 to n-3 fatty acids in the culture medium, confluent BEND cells were incubated with a combination of EPA and increasing ratios of LA to EPA for 24 h (Figure 1B). The total concentration of fatty acids in the culture medium was maintained at 100 μ M. After incubation, the medium was removed, and the cells were incubated in fresh serum-free medium with PDBu (100 ng/mL) for an additional 6 h. Aliquots (0.5 mL) of cell-conditioned media then were collected and stored at -20°C for subsequent PGF $_{2\alpha}$ radioimmunoassay. The remaining cell monolayers were rinsed in ice-cold HBSS, lysed with TriZol, and stored at -80°C until PGHS-2 mRNA quantification.

PGF $_{2\alpha}$ Radioimmunoassay

Prostaglandin F $_{2\alpha}$ concentration in cell-conditioned media was measured in duplicates as described by Darnet-Desnoyers et al. (1994) and modified by Binelli et al. (2000). Assay sensitivity was 5 ng/mL, and intra- and interassay coefficients of variation were 8.2 and 15.8%, respectively. To adjust for between-well differences in cell density, final PGF $_{2\alpha}$ concentrations were expressed as picograms per million cells. Cell numbers in individual dishes were determined using a hemocytometer (Sigma Chemical Co.).

RNA Isolation and Analysis

Total cellular RNA was isolated from control and treated BEND cell cultures using TriZol reagent ac-

Figure 1. Experimental manipulations to study the effects of eicosapentaenoic acid (EPA; A) and linoleic acid (LA; B) on PGF_{2α} production in cultured bovine endometrial (BEND) cells. PDBu = phorbol 12,13-dibutyrate.

cording 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 (Balaguer et al., 2005). After hybridization, RNA filters were washed for 20 min in 50 mL of 2 $\times$ saline sodium citrate, 0.1% SDS at room temperature, followed by two 15-min washes in 0.1 $\times$ saline sodium citrate, and 0.1% SDS at 42°C. The filters were blotted

Figure 2. Effect of eicosapentaenoic acid (EPA) on PGF_{2α} response to phorbol 12,13-dibutyrate (PDBu) in bovine endometrial cells. Data represent least squares means $\pm$ SEM of 3 independent experiments. ^{a-c}Different letters above histograms indicate differences among treatments ($P < 0.05$).

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.

Statistical Analyses

Concentrations of PGF_{2α} in cell culture medium and mRNA responses were analyzed using the GLM procedure of the SAS software package (SAS Institute Inc., Cary, NC). For PGF_{2α} concentration, the sources of variation included experiment, treatment, experiment $\times$ treatment interaction, and well (experiment $\times$ treatment). The well, nested within experiment and treatment, was considered a random variable; therefore, the well 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 mathematical 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. When treatment effects were detected ($P < 0.05$), means were separated using orthogonal contrasts.

RESULTS

Prostaglandin F_{2α} production was negligible in control BEND cells (Figure 2). Treatment with PDBu for 6 h stimulated ($P < 0.01$) PGF_{2α} secretion 10-fold (Figure 2). Preincubation of BEND cells with EPA for 24 h decreased ($P < 0.01$) PGF_{2α} response to PDBu by 75% (Figure 2). However, concentration of PGF_{2α} remained

Figure 3. Effect of eicosapentaenoic acid (EPA) on prostaglandin endoperoxide synthase-2 (PGHS-2) mRNA response to phorbol 12,13-dibutyrate (PDBu) in bovine endometrial cells. Ten micrograms of total cellular RNA isolated from control and treated BEND cells was subjected to Northern blot analysis (A), and resulting densitometric values were analyzed by the GLM procedure of SAS (SAS Institute, Inc., Cary, NC; B). The top panel shows a representative Northern blot; the bottom panel represents means $\pm$ SEM calculated over 2 experiments ($n = 4$ for each treatment). ^{a,b}Different letters above histograms represent differences among treatments ($P < 0.05$).

higher (+2.7-fold) in EPA-treated cells than in control cells (Figure 2).

Compared with untreated cells, PDBu increased ($P < 0.05$) steady-state concentrations of mRNA for PGHS-2 and PPAR δ by 20- and 1.3-fold, respectively (Figures 3 and 4). Eicosapentaenoic acid had no detectable effects on PGHS-2 mRNA or PPAR δ mRNA responses to PDBu (Figures 3 and 4).

The inhibitory effect of EPA on PGF_{2α} production decreased ($P < 0.05$) from 88 to 40% as the ratio of n-6 to n-3 fatty acids in the incubation medium increased from 0 to 19 (Figure 5). Addition of LA alone to the culture medium had no detectable effect on PGF_{2α} response to PDBu (Figure 5). Steady-state PGHS-2 mRNA concentrations increased ($P < 0.05$) from 18 to 93% as the ratio of n-6 to n-3 fatty acids in the culture medium increased from 0 to 19 (Figure 6). Complete substitution of EPA by LA in the medium further enhanced ($P < 0.05$) PGHS-2 mRNA response to PDBu (+149%; Figure 6).

Figure 4. Effect of eicosapentaenoic acid (EPA) on peroxisome proliferator-activated receptor delta (PPAR δ) mRNA response to phorbol 12,13-dibutyrate (PDBu) in bovine endometrial (BEND) cells. Ten micrograms of total cellular RNA isolated from control and treated BEND cells was subjected to Northern blot analysis (A), and resulting densitometric values were analyzed by the GLM procedure of SAS (SAS Institute, Inc., Cary, NC; B). The top panel shows a representative Northern blot; the bottom panel represents means $\pm$ SEM calculated over 2 experiments ($n = 4$ for each treatment). ^{a,b}Different letters above histograms represent differences among treatments ($P < 0.05$).

DISCUSSION

Evidence is rapidly accumulating that supplemental fatty acids can have major effects on eicosanoid synthesis in domestic animals (Baguma-Nibasheka et al., 1999; Cheng et al., 2001; Chartrand et al., 2003; Mattos et al., 2003). Depending on the amount of particular fatty acids reaching the target tissues, supplemental fatty acids can either stimulate (Burke et al., 1996; Filley et al., 1999) or inhibit (Baguma-Nibasheka et al., 1999; Cheng et al., 2001; Mattos et al., 2003) prostanoid synthesis. Results of this study extend previous observations that n-3 fatty acids are potent inhibitors of PG secretion in mammalian species (Olsen et al., 1992; Baguma-Nibasheka et al., 1999; Mattos et al., 2000; Cheng et al., 2001; Mattos et al., 2002; Mattos et al., 2003). Although the exact mechanism by which supplemental n-3 fatty acids inhibit PGF_{2α} production is not fully understood, it is conceivable that increased avail-

Figure 5. Effect of increasing the ratio of n-6 to n-3 fatty acids (n-6/n-3) on PGF_{2α} response to phorbol 12,13-dibutyrate (PDBu). Data represent least squares means $\pm$ SEM of 3 independent experiments. Different letters above histograms indicate differences among treatments ($P < 0.05$). LA = linoleic acid; EPA = eicosapentaenoic acid. ^{a-c}Different letters above histograms indicates differences among treatments ($P < 0.05$).

ability of EPA in membrane phospholipids as a result of treating BEND cells with EPA could displace ARA, leading to increased synthesis of PG of the 3 series at the expense of PG of the 2 series (Mattos et al., 2003). This hypothesis does not rule out the possibility that supplemental EPA also may inhibit PGHS-2 activity in cultured BEND cells. In fact, incubation of rat hepatoma cells with ARA, EPA, DHA, or heineicosapentaenoic acid (C_{21:5} n-3) inhibited PGHS-2 enzyme activity (Larsen et al., 1997). Eicosapentaenoic acid inactivated the enzyme almost completely when added 30 s before the addition of ARA.

The rate-limiting step in PG synthesis involves the cleavage of sn-2 fatty acyl ester bond of membrane phospholipids by cytosolic phospholipase A₂ (Van den Bosch, 1980; Irvine, 1982). The ARA that is released by phospholipid hydrolysis is acted on by PGHS-2 to form PGH₂, which then is converted to PGF_{2α}. Consistent with a previous observation (Mattos et al., 2003), the present study provided no evidence for EPA regulation of PGHS-2 mRNA abundance in BEND cells. As discussed previously, supplemental EPA may alter endometrial PGF_{2α} production through competitive displacement of ARA from membrane phospholipids, or through posttranslational alteration of the PGHS-2 enzymatic activity, or both.

Polyunsaturated fatty acids elicit several physiological changes through the alteration of the activity or

Figure 6. Effects of increasing the ratio of n-6 to n-3 fatty acids (n-6/n-3) on prostaglandin endoperoxide synthase-2 (PGHS-2) response to phorbol 12,13-dibutyrate (PDBu). Ten micrograms of total cellular RNA isolated from control and treated bovine endometrial cells were subjected to Northern blot analysis (A), and resulting densitometric values were analyzed by the GLM procedure of SAS (SAS Institute, Inc., Cary NC; B). The top panel shows a representative Northern blot; the bottom panel represents means $\pm$ SEM calculated over 2 experiments ($n = 4$ for each treatment). ^{a-c}Different letters above histograms represent differences among treatments ($P < 0.05$). LA = linoleic acid; EPA = eicosapentaenoic acid.

synthesis of nuclear PPAR (Bocher et al., 2002). In mice, PPAR δ deficiency leads to placental defects and results in frequent mid gestational lethality (Barak et al., 2002), suggesting that this nuclear receptor may play an important role in the control of reproductive processes in mammalian species. Consistent with a recent in vivo experiment (Palin et al., 2005), supplemental EPA had no detectable effects on PPAR δ mRNA response to PDBu in cultured BEND cells. Results suggest that supplemental n-3 fatty acids likely alter endometrial PGF $_{2\alpha}$ production through a mechanism that does not require induction of the PPAR δ gene. However, whether and how these fatty acids may control the activity of this nuclear receptor warrants further investigation.

Conventional cattle diets contain a mixture of n-6 and n-3 fatty acids. Therefore, we examined the effect of EPA on endometrial production of PGF $_{2\alpha}$ in the pres-

ence of increasing concentrations of LA. The inhibitory effect of EPA on uterine endometrial PGF $_{2\alpha}$ production decreased from 88 to 40% as the ratio of n-6 to n-3 fatty acids in the culture medium increased from 0 to 19. These findings are consistent with previous reports (Trujillo and Broughton, 1995; Achard et al., 1997) and suggest that the net inhibition of uterine PGF $_{2\alpha}$ synthesis by n-3 fatty acids may depend on the amount of n-6 fatty acids reaching the target tissue. Increasing the ratio of n-6 to n-3 fatty acids in the incubation medium may increase the availability of ARA in membrane phospholipids and, therefore, decrease the competition by n-3 fatty acids for the PGHS-2 enzyme.

CONCLUSIONS

Results provide further evidence for phorbol ester stimulation of PGF $_{2\alpha}$ production in cultured BEND cells. Preincubation of confluent BEND cells with EPA for 24 h decreased PGF $_{2\alpha}$ response to PDBu, but had no detectable effect on PGHS-2 mRNA abundance. The inhibitory effect of EPA on PGF $_{2\alpha}$ production was reduced in BEND cells treated with increasing ratios of LA to EPA, suggesting that the net inhibition of uterine endometrial synthesis by n-3 fatty acids may vary depending on the ratio of n-6 to n-3 fatty acids in the uterus. Whether and how these fatty acids alter PGHS-2 activity is yet to be elucidated.

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