Polyunsaturated Fatty Acids and Bovine Interferon-τ Modify Phorbol Ester-Induced Secretion of Prostaglandin F2α and Expression of Prostaglandin Endoperoxide Synthase-2 and Phospholipase-A2 in Bovine Endometrial Cells

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ABSTRACT

Embryonic mortality in cattle may occur because of inadequate inhibition of uterine secretion of prostaglandin (PG) F2α mediated by bovine interferon-τ (bIFN-τ). The objectives of the present study were to determine whether polyunsaturated fatty acids inhibit secretion of PGF2α from bovine endometrial cells induced by stimulating protein kinase C with phorbol 12,13-dibutyrate (PDBu) and to investigate possible mechanisms of action. Confluent cells were exposed for 24 h to 100 μM of linoleic, arachidonic (AA; C20:4, n-6), linolenic (LNA; C18:3, n-3), eicosapentaenoic (EPA; C20:5, n-3), or docosahexaenoic (DHA; C22:6, n-3) acid. After incubation, cells were washed and stimulated with PDBu. The EPA, DHA, and LNA attenuated secretion of PGF2α in response to PDBu. The EPA and DHA were more potent inhibitors than LNA. The EPA inhibited secretion of PGF2α at 6.25 μM. Secretion of PGF2α in response to PDBu decreased with increasing incubation time with EPA. Both bIFN-τ and EPA inhibited secretion of PGF2α, and their inhibitory effects were additive. The bIFN-τ, but not EPA, reduced the abundance of PG endoperoxide synthase-2 (PGHS-2) mRNA. Incubation with 100 μM EPA, DHA, or AA for 24 h followed by treatment with PDBu did not affect concentrations of PGHS-2 and phospholipase A2 proteins. The EPA and DHA inhibit secretion of PGF2α through a mechanism different from that of bIFN-τ. The effect of EPA on PGF2α secretion may be caused by competition with AA for PGHS-2 activity or reduction of PGHS-2 activity. The use of EPA and DHA to inhibit uterine secretion of PGF2α to improve embryonic survival in cattle warrants further investigation.

INTRODUCTION

A high proportion of embryonic loss occurs in cattle during the first 3 wk of pregnancy [1]. This high proportion of losses coincides with the period of embryonic inhibition of uterine prostaglandin (PG) F2α secretion and suggests that some loss may be occurring because certain embryos are unable to inhibit secretion of PGF2α. Therefore, strategies to further inhibit secretion of PGF2α may increase embryonic survival. In a recent report, increased PG synthesis induced by oxytocin during Days 5–8 of pregnancy reduced the pregnancy rates of beef cows at 30 days after artificial insemination from the 80% observed in the untreated controls to 30%. Treating cows concomitantly with flunixin meglumine, an inhibitor of prostanoïd synthesis, restored pregnancy rates to 80% [2]. Another study [3] demonstrated that chronic treatment with PGF2α reduced the pregnancy rate when given between Days 5 and 8 of pregnancy and not on Days 10–13 and 15–18. These results demonstrate that increased PG secretion during early pregnancy causes embryonic loss. In addition, the results support the hypothesis that reducing PG synthesis during early pregnancy reduces embryonic loss and improves pregnancy rates.

Secretion of prostanoids, such as PGF2α, requires a cascade of intracellular events, including activation of the phospholipase (PL) C enzyme and activation of a calcium-dependent PLA2 through a multiple antigenic peptide kinase-dependent pathway [4]. The activated PLA2 translocates to the plasma membrane and cleaves fatty acids from the sn-2 position of membrane phospholipids, to which a high proportion of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), is esterified. Released AA is converted to PGH2 by the PG endoperoxide H synthase (PGHS) enzymes. Two PGHS enzymes, PGHS-1 and PGHS-2, with similar structures and functions but different expression patterns, have been described [5]. Prostaglandin H2 is subsequently reduced to PGF2α by PGF2α synthase or isomerized to other prostanoïds of the 2 series, such as PGE2 [6].

The PLA2 and PGHS enzymes also are capable of processing other fatty acids, such as eicosapentaenoic acid (EPA), which is the precursor for synthesis of prostanoïds of the 3 series. Prostanoids of the 3 series, which include PGF3 and PGE3, have three double bonds in the carbon chain, as opposed to the two double bonds found in prostanoïds of the 2 series. Increased availability of n-3 PUFAs, such as EPA and docosahexaenoic acid (DHA), in membrane phospholipids could inhibit PGF2α secretion through three mechanisms. First, EPA could displace AA, leading to increased synthesis of prostanoïds of the 3 series at the expense of prostanoïds of the 2 series, such as PGF2α. Second, EPA and another n-3 PUFA, DHA, may reduce expression of the PGHS enzymes [7], which could make these enzymes less available and further reduce prostanoïd synthesis. Third, the conversion of EPA into prostanoïds of the 3 series is less efficient than the conversion of AA into prostanoïds of the 2 series [8]. A lower efficiency of catalysis may result in reduced total prostanoïd synthesis because of insufficient converting capacity.

Prostanoids of the 3 series are less bioactive [9], and their role in ruminant luteolysis is currently unknown. Prostaglandin F3α has only 25% affinity for the ovine luteal PGF2α receptor when compared to PGF2α [10]. In addition,
DHA is not a substrate for the PGHS enzymes but is a strong competitive inhibitor of their activity [11]. The bovine interferon-τ (bIFN-τ) attenuates secretion of endometrial PGF₂ₐ from both in vivo [12] and in vitro [13–16]. Experimental approaches used to test the effects of bIFN-τ have included explants [13], primary cultures of endometrial cells collected on Days 1–4 [15] or on Day 15 of the estrous cycle [14], and bovine immortalized endometrial (BEND) cells [16]. The BEND cells are a line of spontaneously replicating endometrial cells originating from Day 14 cycling cows [17]. Systems used to evaluate secretion of PGF₂ₐ from endometrial cells generally involve stimulation of cells with an activator of protein kinase C, such as phorbol 12-myristate 13-acetate and phorbol 12,13 di-butyrat (PDBu) [16]. These activators induce expression of the PGHS-2 gene and stimulate secretion of PGF₂ₐ. The bIFN-τ attenuates both of these effects [15, 16].

The objectives of the present study were to determine the effects of PUFA s on secretion of PGF₂ₐ from BEND cells stimulated with PDBu, to test the interaction of PUFA s with the inhibitory effect of bIFN-τ, and to explore possible mechanisms of action by determining the effects of PUFA s on steady-state concentrations of PGHS-2 mRNA and protein expression of PLA₂ and PGHS-2.

**MATERIALS AND METHODS**

**Materials**

Recombinant bIFN-τ (1.08 × 10⁷ U of antiviral activity per milligram) was donated kindly by Dr. R. Michael Roberts (University of Missouri, Columbia, MO). Polystryene tissue-culture Costar six-well plates and culture dishes (100 × 20 mm) were purchased from Corning (Corning Glass Works, Corning, NY). Polystyrene flasks for cell culture (175 cm²) were from Sarstedt, Inc. (Newton, NC). Acrylamide, N,N'-methylenebisacrylamide, and Nonidet 40 were from BDH Laboratory Supplies (Poole, U.K.).

The Ham F-12, Eagle minimum essential medium (MEM), antibiotic-antimycotic solution (AbAm), 3-(N-Morpholino) propanesulfonic acid (MOPS), and EDTA were from Sigma Chemical Co. (St. Louis, MO). The lysis buffer was from Promega (Reportor lysis buffer; Madison, WI). Isotopically labeled [5, 6, 8, 11, 12, 14, 15, 16]-H-PGF₂α (212 Ci/mole), and nitricellulose membranes (Hybond-ECL) were from Amersham Pharmacia Biotech (Arlington Heights, IL). BioTrans Nylon membrane was from ICN Chemicals (Irvine, CA). Hanks balanced salt solution (HBSS) and TRIzol were from Life Technologies (Grand Island, NY). Polystyrene flasks for cell culture (175 cm²) were purchased from Corning (Corning Glass Works, Corning, NY). Polystyrene dishes (24 × 10³ cell/dish) for experiments 7 and 8. The ratio of culture area to volume of culture medium was the same in both culture containers. Cells were grown to confluence at 37°C in an incubator (model MCO 17AI; Sanyo Electric Co., Ltd., Osaka, Japan) under a humidified atmosphere containing 95% air and 5% CO₂. On reaching confluence, cells were washed twice with HBSS at 37°C and cultured for an additional 24 h in culture medium devoid of serum with or without fatty acids and PDBu. The 24-h incubation period with or without fatty acids and exposed to serum-free medium mixed with appropriate treatments. In experiments 1–6, samples (250 µl) were collected immediately before cell wash (0 h) and at 3 and 6 h after addition of PDBu. At 3 h, the volume was removed and replaced by adding 250 µl of medium devoid of serum with PDBu (100 ng/ml). In experiments 7–9, samples of medium were collected at 6 h only. After collection, all samples were stored at −20°C until analyzed for PGF₂α.

**Preparation of Fatty Acid Solutions**

Stock solutions of polyunsaturated fatty acids were diluted in ethanol (50 mg/ml), aliquoted, and stored at −80°C. At preparation of culture medium with fatty acid, the ethanol was evaporated with nitrogen gas, and the remaining concentrated fatty acid solution was diluted to 1 µM in culture medium (50% MEM and 50% Ham F-12) containing 33 mg/ml of fatty acid-free BSA (fraction V, low endotoxin). This solution was incubated for 2 h in a water bath at 37°C to allow binding of the fatty acid to BSA and further diluted in culture medium to final treatment concentrations. The BSA:fatty acid ratio was maintained constant at all fatty acid concentrations tested.

**Experiment 1.** The objective of this experiment was to test the effect of different PUFA s and BSA on secretion of PGF₂α induced by PDBu. Cultures (3 ml of culture medium, six-well plates, n = 30) were assigned randomly to incubation for 24 h with medium devoid of serum with or without fatty acid, medium devoid of serum with BSA (3.3 mg/ml), or medium devoid of serum with BSA and one of the following fatty acids (100 µM): EPA, DHA, AA, LNA, LA, or OA.Thirty 35-mm wells were assigned to each treatment. After the 24-h incubation, cells were washed with HBSS and exposed to culture medium containing PDBu (100 ng/ml). The control groups included cells incubated with BSA that subsequently were exposed or not exposed to PDBu and cells incubated for 24 h with culture medium alone (no BSA) and subsequently stimulated with PDBu.

**Experiment 2.** This experiment was conducted to test the effect of different concentrations of EPA, DHA, and LNA on PGF₂α secretion from BEND cells stimulated with PDBu. Cultures (3 ml of culture medium, six-well plates, n = 54) were assigned in triplicate to incubation for 24 h in medium devoid of serum with treatments of a 3 × 6 factorial experiment. Factors were fatty acid (EPA, DHA, or LNA) and concentration (0, 20, 40, 60, 80, or 100 µM). At 0 h, cells were washed twice with HBSS and treated with medium devoid of serum containing PDBu (100 ng/ml).

**Experiment 3.** This experiment was conducted to test the effect of combinations of EPA between 0 and 20 µM on PGF₂α secretion from BEND cells stimulated with PDBu. Cultures (3 ml of culture medium, six-well plates, n = 12) were assigned in triplicate to incubation with medium devoid of serum with BSA and 0, 6.25, 12.5, or 20 µM EPA for 24 h and subsequently washed and stimulated with PDBu (100 ng/ml) dissolved in medium devoid of serum.

**Experiment 4.** This experiment was conducted to test the effect of different concentrations of incubation on PGF₂α secretion from BEND cells stimulated with PDBu. Cultures (3 ml of culture medium, six-well plates, n = 18) were assigned in triplicate to incubation with medium devoid of serum with BSA and 100 µM EPA for 0, 1, 3, 6, 12, or 24 h. After incubation, cells were washed and treated with PDBu (100 ng/ml) dissolved in culture medium devoid of serum.

**Experiment 5.** This experiment was conducted to test the effect of concentrations of bIFN-τ between 0 and 25 ng/ml on PGF₂α secretion from BEND cells stimulated with PDBu. Cultures (3 ml of culture medium, six-well plates, n = 27) were incubated for 24 h with medium devoid of serum without fatty acids. After incubation, cells were washed and assigned in triplicate to receive medium devoid of serum containing PDBu (100 ng/ml) in combination with 0, 0.01, 0.05, 1, 2, 3, 12.5, or 25 ng/ml of bIFN-τ.

**Experiment 6.** This experiment was conducted to test the effect of combinations of different doses of EPA and bIFN-τ on secretion of PGF₂α from BEND cells stimulated with PDBu. Cultures (24 ml of culture medium, six-well plates, n = 27) were assigned in triplicate to a 3 × 3 factorial experiment. Factors were incubated for 24 h with medium devoid of serum, and the remaining containing EPA and subsequent treatment with bIFN-τ (0, 50, or 100 pg/ml) given concurrently with PDBu (100 ng/ml). At the end of incubation with EPA, cells were washed twice with HBSS.
to remove EPA and BSA and treated with PDBu (100 ng/ml) dissolved in medium devoid of serum and containing the different concentrations of bIFN-α. Whole-cell extracts (WCEs) were collected at 6 h to determine protein yields. This experiment was replicated once.

Experiment 7. This experiment was conducted to compare the effect of three concentrations of EPA, DHA, and AA on secretion of PGF2α. Cells were incubated for 24 h with medium devoid of serum containing 0, 20, or 100 μM EPA followed by treatment with 100 ng/ml of PDBu in serum-free medium without fatty acids or incubation for 24 h with serum-free medium without fatty acids followed by treatment at 0 h with 100 ng/ml of PDBu in combination with 0, 0.5, or 50 ng/ml of bIFN-α. Total RNA extraction was conducted at 6 h. This experiment was replicated once.

Experiment 8. This experiment was conducted to test the effect of different concentrations of EPA, DHA, and AA on secretion of PGF2α, and intracellular concentrations of PGHS-2 and cPLA2 protein in BEND cells stimulated with PDBu. Cultures (24 ml of culture medium, 100-mm dishes, n = 12) were assigned in duplicate to incubation for 24 h with medium devoid of serum containing 100 μM EPA, DHA, or AA. After incubation, cells were washed twice with HBSS and treated with 100 ng/ml of PDBu dissolved in medium devoid of serum. The WCEs were collected at 6 h after addition of PDBu for PGHS-2 and cPLA2 immunoblotting. This experiment was replicated once.

Experiment 9. This experiment was conducted to test the effect of combinations of EPA, DHA, and AA concentrations on secretion of PGF2α from BEND cells stimulated with PDBu. Cultures (3 ml of culture medium, six-well plates, n = 27) were assigned in triplicate to a 3 × 3 factorial experiment. Cells were incubated for 24 h with medium devoid of serum containing all factorial combinations of EPA (0, 25, and 100 μM) and AA (0, 25, and 100 μM). After incubation, cells were washed twice with HBSS and treated with 100 ng/ml of PDBu dissolved in medium devoid of serum. Medium samples were at 6 h after PDBu.

Preparation of Cell Extracts and Immunoblotting Analysis

The WCEs were harvested immediately after collection of the last medium sample. Cells were washed twice with HBSS at 4°C and incubated for 15 min with 1 ml of lysis buffer. Dishes were then scraped using a rubber policeman to collect cell fragments attached to the dish. Lysates and cell fragments were transferred to 1.5-ml conical tubes and agitated for 5 min using a rotating device (Roto-torque; Cole-Parmer Instrument Co., Chicago, IL) to allow further cell lysis. Cell debris was separated by centrifugation using a microcentrifuge (10,000 × g) for 2 min. Supernatants were transferred to 1.5-ml tubes and stored at −20°C. Concentrations of protein in WCEs were determined using the Bradford method [21]. A sample of each WCE was diluted 1:25 (40 μl of sample in 960 μl of ddH2O) and used for determination of protein concentration. Twenty-five microgram of protein were loaded onto duplicate 7.5% acrylamide gels. Twenty-five microgram of protein were loaded onto duplicate 7.5% acrylamide gels. The PGF2α mRNA transcript was identified by autoradiography, and hybridization signals were analyzed by densitometry.

Radioimmunoassay

Concentrations of PGF2α were measured in medium samples collected at 0 and 6 h after treatment of cells with PDBu. Twenty-five microliters of each sample were further diluted in 75 μl of 50 mM Tris-HCl (pH 7.5, Tris buffer). The total volume of diluted samples for assay was 100 μl. Danet-Desnoyers et al. [14] have described the RIA procedure. To prepare standard curves, known amounts of nonradioactive PGF2α (1.25–1000 pg/tube) were diluted in Tris buffer (100 μl). An anti-PGF2α antiserum [22] was diluted 1:5000 (Tris buffer, 100 μl/tube). Final assay volume was 400 μl (100 μl of Tris buffer, 100 μl of diluted sample, 100 μl of antibody solution, and 100 μl of radiolabeled PGF2α solution). The minimum detectable concentration of PGF2α was 3.32 pg/tube. Inter- and intrassay coefficients of variation were 11.2% and 13.3%, respectively. Assay was validated for serum-free medium by adding 25 pg of PGF2α per 0.1 ml to medium. Average recovered PGF2α was 25.1 ± 1.42 pg per 0.1 ml, which yielded a calculated recovery of 100.25%. A test using known amounts of PGF2α resulted in a cross-reactivity of 39.8%. Cross-reactivity with PGE2 was less than 1%. Concentrations of PGF2α used in the test of cross-reactivity were 0.625, 1.25, 2.5, 5, 10, and 20 ng/ml.

Concentrations of PGF2α tested were 20, 40, 80, and 100 ng/ml. Concentrations of PGF2α are reported as picograms per milliliter of culture medium because treatments did not affect cell numbers, as determined by protein yield (experiments 6 and 8).

Statistical Analyses

Data were analyzed by least-squares ANOVA using the general linear models procedure of SAS [23]. Data for each experiment were analyzed separately. The PGF2α data were analyzed as a split-plot design. Models included the effects of treatment well (or dish) within treatment, time, interaction between time and treatment, and residual error. The effect of time (or dish) within treatment was used as the error term to test for the effects of treatment. Preplanned orthogonal contrasts and pairwise comparisons were used to identify differences between means. Concentrations of PGF2α in medium at 6 h after addition of PDBu were greater than at 3 h (P < 0.01), and the pattern of responses among treatments were comparable at 3 and 6 h. The 6-h responses for all experiments are reported. For abundance of PGHS-2 and cPLA2, the models included the effect of treatment only. Values of PGF2α mRNA abundance in experiment 7 were adjusted for variability of loading using the abundance of 18s rRNA as a covariate. Preplanned pairwise comparisons were used to determine differences between means.

The test of homogeneity of polynomial regression [24] was used in experiment 2 to compare the inhibition curves obtained using increasing concentrations of EPA, DHA, and LNA.

RESULTS

Effect of Polysaturated Fatty Acids on Secretion of PGF2α

Incubation for 24 h with 100 μM of the omega-3 fatty acids EPA, DHA, and LNA significantly attenuated secretion of PGF2α (P < 0.05) in response to PDBu when compared with incubation without fatty acid (Fig. 1). Arachidonic acid (100 μM) had the opposite effect and tended to increase secretion of PGF2α in response to PDBu (P = 0.1). The other fatty acids tested (100 μM), OA and LA, did not affect production of PGF2α (P > 0.05). Incubation with BSA without fatty acid did not affect secretion of PGF2α in response to treatment with PDBu. Cells incubated with BSA and not stimulated with PDBu did not secrete detectable amounts of PGF2α.

In experiment 2, incubation with increasing concentrations (dose) of n-3 PUFAs resulted in a dose-responsive reduction in secretion of PGF2α (P < 0.001). Treatment with 20 μM of any of the three fatty acids resulted in lower secretion of PGF2α in comparison to the control without fatty acid (P < 0.02). Treatment with EPA [Y = 6078 − (225 × dose) + (3.4 × dose²) − (0.016 × dose³); R² = 0.992], DHA [Y = 6078 − (225 × dose) + (3.4 × dose²) − (0.016 × dose³); R² = 0.987] and LNA [Y = 6078 − (225 × dose) + (3.4 × dose²) − (0.016 × dose³); R² = 0.981] significantly decreased PGF2α secretion in a dose-dependent manner. Statistical analyses were conducted using the least-squares regression method [25].
FATTY ACIDS REGULATE PROSTAGLANDIN SECRETION

FIG. 1. Experiment 1. Least-square means and SEM of concentrations of PGF$_{2a}$ in medium conditioned by BEND cells 6 h after treatment with PDBu (100 ng/ml). Cells were incubated with no fatty acid (Control) or with 100 μM EPA, DHA, AA, LA, LNA, or OA for 24 h before treatment with PDBu. A second control group was included to test the effect of BSA used to solubilize the fatty acids. The BSA did not affect PGF$_{2a}$ secretion. Differences between different fatty acids and the Control: *$P < 0.10$, **$P < 0.01$. and ***$P < 0.001$.

FIG. 2. Experiment 2. Prostaglandin F$_{2a}$ response curves for increasing concentrations (0, 20, 40, 60, 80, or 100 μM) of the fatty acids EPA, DHA, or LNA. Concentrations were measured in medium conditioned by BEND cells 6 h after treatment with PDBu (100 ng/ml). Cells were incubated for 24 h before treatment with PDBu in a 3 × 6 factorial design. Both EPA and DHA were more potent inhibitors than LNA ($P < 0.01$).

FIG. 3. Experiment 4. Least-square means and SEM of concentrations of PGF$_{2a}$ in medium conditioned by BEND cells 6 h after treatment with PDBu (100 ng/ml). Cells were incubated with the fatty acid EPA (100 μM) for 0 (Control), 1, 3, 6, 12, or 24 h before treatment with PDBu. Secretion of PGF$_{2a}$ in response to PDBu decreased with increasing incubation time ($P < 0.01$). Differences between different incubation times and the Control: *$P < 0.1$, **$P < 0.01$, and ***$P < 0.001$. of the cells to the medium containing 100 μM EPA ($P < 0.01$, experiment 4; Fig. 3).

Treatment of cells with PDBu in combination with increasing concentrations of biIFN-τ (experiment 5) resulted in a dose-dependent decrease in concentrations of PGF$_{2a}$ ($P < 0.001$). Concentrations of PGF$_{2a}$ in the medium of cells treated with biIFN-τ at 0.01, 0.1, 0.5, 1, 2, 3, 12.5, 12.5, or 20 ng/ml were 6570, 4485, 3314, 2388, 1793, 966, and 1131 pg/ml (SEM = 398), respectively. Inhibition of PGF$_{2a}$ secretion was significant at all concentrations of biIFN-τ tested when compared to the control without biIFN-τ ($P < 0.01$).

In Experiment 6, both incubation for 24 h with EPA ($P < 0.002$) and subsequent treatment with biIFN-τ ($P < 0.001$) inhibited secretion of PGF$_{2a}$ in response to PDBu (Fig. 4). No interaction between biIFN-τ and EPA was detected, indicating that EPA and biIFN-τ acted in an additive manner. Treatments did not affect concentrations of protein measured in WCEs collected at 6 h.

FIG. 4. Experiment 6. Least-square means and SEM of concentrations of PGF$_{2a}$ in medium conditioned by BEND cells 6 h after treatment with PDBu (100 ng/ml). Cells were incubated with the fatty acid EPA (100 μM) for 0 (Control), 1, 3, 6, 12, or 24 h before treatment with PDBu. Secretion of PGF$_{2a}$ in response to PDBu decreased with increasing incubation time ($P < 0.01$). Differences between different incubation times and the Control: *$P < 0.1$, **$P < 0.01$, and ***$P < 0.001$.
Effects of Polyunsaturated Fatty Acids and bIFN-τ on Steady-State Concentrations of PGHS-2 mRNA: PGHS-2 Northern Blot Analysis

Incubation of cells with EPA for 24 h did not affect PGHS-2 mRNA concentrations (P > 0.1). However, bIFN-τ reduced PGHS-2 mRNA concentrations in a dose-responsive manner (P < 0.01, experiment 7; Fig. 5). Reduction of PGHS-2 mRNA abundance relative to the control not treated with bIFN-τ was 26.5% and 90.7% for 0.5 and 50 ng/ml of bIFN-τ, respectively. Both EPA and bIFN-τ attenuated the secretion of PGF₂α, at 6 h after addition of PDBu (P < 0.01). Concentrations of PGF₂α in medium from controls (no EPA and no bIFN-τ) were greater (5504.3 ± 217 pg/ml, P < 0.01) than those in medium from cells treated with 20 μM EPA (3100 ± 307 pg/ml), 100 μM EPA (1777 ± 307 pg/ml), 0.5 ng/ml of bIFN-τ (2240 ± 307 pg/ml), or 50 ng/ml of bIFN-τ (334 ± 307 pg/ml).

Effects of Polyunsaturated Fatty Acids on PGHS-2 and cPLA₂ Protein Expression: PGHS-2 and PLA₂ Immunoblotting

Concentrations of PGF₂α in medium at 6 h after PDBu were increased by incubation for 24 h with AA (1991 ± 84.2 pg/ml, P < 0.01) and decreased (P < 0.01) by EPA (567 ± 84.2 pg/ml) and DHA (756 ± 84.2 pg/ml) relative to the control with PDBu alone (1454 ± 84.2 pg/ml). Concentrations of PGHS-2 were not affected by any of the fatty acids tested (P > 0.05). Treatment with DHA tended to reduce the abundance of PLA₂ (P = 0.08, experiment 8; Fig. 6). Treatments did not affect concentrations of protein measured in WCEs collected at 6 h.

Effect of Coincubation with AA and EPA on PGF₂α Secretion

Coincubation of BEND cells with AA and EPA for 24 h (experiment 9) demonstrated the competing effects of the...
two fatty acids. Arachidonic acid increased \( (P < 0.01) \) secretion of PGF\(_{2\alpha}\) in response to PDBu, whereas EPA was inhibitory \( (P < 0.01; \text{Fig. 7}) \). The response to incubation with 25 \( \mu \text{M} \) AA tended to decrease with increasing concentrations of EPA \( (P = 0.09, \text{EPA} \times \text{AA interaction}) \). Incubation with 100 \( \mu \text{M} \) EPA resulted in a 72.4% (2198 pg/ml) inhibition of PGF\(_{2\alpha}\) secretion relative to the untreated control (7960 pg/ml), but this inhibitory effect was reversed with coinubcation of EPA (100 \( \mu \text{M} \)) with AA (100 \( \mu \text{M} \)), causing the concentrations of PGF\(_{2\alpha}\) in the medium to be similar to that of the control (6715 pg/ml, \( P > 0.05 \)).

### DISCUSSION

The experiments described indicate that PUFAs can modify the secretion of PGF\(_{2\alpha}\), in a model in which BEND cells are stimulated with PDBu. Binelli et al. [16] reported that PDBu (100 ng/ml) increases steady-state concentrations of PGHS-2 mRNA and protein and induces secretion of PGF\(_{2\alpha}\). Moreover, it was determined that bIFN-\(\tau\) (50 ng/ml) suppresses PGF\(_{2\alpha}\), probably through reduction of PGHS-2 mRNA concentrations, protein expression, and enzymatic activity of PGHS-2 and PLA\(_2\). A control without PDBu was run only in experiment 1, because that experiment demonstrated that incubation of cells with PUFA did not induce secretion of PGF\(_{2\alpha}\). Concentrations of PGF\(_{2\alpha}\) were undetectable in that group. In a previous report [16], maximal induction of PGF\(_{2\alpha}\) secretion was detected between 3 and 6 h after treatment of cells with PDBu and coincided temporarily with an increase in the intracellular mass of PGHS-2 protein. Based on these results, the present experiments were designed to determine the effects of fatty acids on secretion of PGF\(_{2\alpha}\) and enzyme expression (PGHS-2 and PLA\(_2\)) at 6 h after treatment with PDBu.

The antibody used to determine concentrations of PGF\(_{2\alpha}\) also had a significant cross-reactivity (39.8%) for PGF\(_{3\alpha}\). Although we were unable to separate the contribution of PGF\(_{3\alpha}\) to the PGF\(_{2\alpha}\) measurements, any increased production of PGF\(_{3\alpha}\) as a result of incubation with EPA or other n-3 fatty acids would have reduced the apparent differences of PGF\(_{2\alpha}\) concentrations measured by RIA between cells treated with PDBu and EPA and cells treated with PDBu alone. In other words, if EPA or other n-3 fatty acids indeed increased production of PGF\(_{3\alpha}\), then the real differences in production of PGF\(_{2\alpha}\) between controls treated with PDBu alone and cells incubated with EPA or other n-3 fatty acids were greater than those detected by the RIA.

Incubation of cells with culture medium containing a fatty acid bound to BSA increases the concentration of that particular fatty acid in the plasma membrane [25]. Experiment 4 demonstrated that the inhibitory effect of EPA on secretion of PGF\(_{2\alpha}\) increased with longer exposure of cells to EPA. Although incorporation of fatty acids to the plasma membrane was not measured in the present study, the mass of EPA incorporated to the plasma membrane likely increased with increasing incubation time, and this greater incorporation likely resulted in greater inhibition of PGF\(_{2\alpha}\) secretion. It is possible that longer incubation periods would result in a greater inhibition of PGF\(_{2\alpha}\) secretion. Therefore, it is also possible that the effects described in experiments 1–3 and 6–9 could have been attained using lower doses and longer incubation times.

In experiments 1 and 2, incubation of BEND cells with the n-3 PUFAs EPA, DHA, and LNA caused a dose-dependent reduction of PGF\(_{2\alpha}\) secretion in response to stimulation with PDBu. Incubation for 24 h with EPA was significant even at the lowest concentration tested (6.25 \( \mu \text{M}, \text{experiment 3}) \). These findings are in agreement with reports indicating that PUFAs of the n-3 and n-6 families can inhibit the secretion of prostanoids in several cell types cultured in vitro [7, 26]. Other studies have demonstrated reduced prostanoid synthesis when fatty acids of the n-3 and n-6 families are fed in the diet [27]. It is not clear why LNA was less inhibitory than DHA and EPA. Linoleic acid is the precursor for synthesis of DHA and EPA and can be converted to them in a process that relies on activities of desaturase and elongase enzymes. It is possible that some conversion of LNA to EPA and DHA occurred during the period of incubation, leading to the observed reduction in PGF\(_{2\alpha}\) secretion. The fact that LA did not affect secretion of PGF\(_{2\alpha}\) in response to PDBu somewhat contradicts previous reports indicating an inhibitory activity of this fatty acid [28, 29]. Moreover, LA has been considered to be a potential modulator of reduced endometrial PGF\(_{2\alpha}\) synthesis in the pregnant cow [1]. Conversely, because LA is the most abundant precursor for synthesis of AA and PGF\(_{2\alpha}\), it could be hypothesized that LA would increase secretion of PGF\(_{2\alpha}\) through increased precursor availability. This did not occur in the BEND cell system. Possible reasons could involve lack of an efficient system for conversion of LA to AA, which involves two steps of desaturation and one step of elongation. The fact that incubation with 100 \( \mu \text{M} \) AA increased production of PGF\(_{2\alpha}\) (experiments 1 and 8) supports the concept that availability of AA limits secretion of PGF\(_{2\alpha}\) and that conversion of LA to AA does not occur efficiently in the cell culture system used. Oleic acid was included as a fatty acid control that is not a substrate for the PGHS enzymes and does not interfere with synthesis of prostanoids [7]. Oleic acid did not affect secretion of PGF\(_{2\alpha}\) in response to stimulation with PDBu.

Experiment 6 demonstrated that EPA can amplify the inhibitory effects of bIFN-\(\tau\) on secretion of PGF\(_{2\alpha}\) in an additive manner. The concentrations of EPA and bIFN-\(\tau\) included in experiment 6 were based on the results of experiments 3 and 5, which determined concentrations of EPA and bIFN-\(\tau\) that partially inhibit secretion of PGF\(_{2\alpha}\). Utilizing these partially inhibitory doses, a significant amplification in PGF\(_{2\alpha}\) inhibition was detected in experiment...
6 by addition of an equivalent dose of the other inhibitor. The implication of this finding is that supplementation with inhibitory fatty acids, such as EPA, during early pregnancy by dietary or parenteral means may further enhance the suppression of PGF\(_{2\alpha}\) secretion in concert with the action of embryonic bIFN-\(\tau\). Because a significant proportion of bovine embryos is thought to be lost because of inadequate inhibition of uterine PGF\(_{2\alpha}\) secretion, further inhibition by exogenous means may result in increased embryo survival. This hypothesis is supported by the study of Burke et al. [30], in which feeding a source of EPA and DHA, fish meal, to lactating dairy cows resulted in increased pregnancy rates.

Results from experiments 7 and 8 indicated that EPA and bIFN-\(\tau\) inhibit secretion of PGF\(_{2\alpha}\) through different mechanisms. Bovine interferon-\(\tau\), but not EPA, reduced steady-state concentrations of PGHS-2 mRNA. In experiment 7, the degree of inhibition relative to the control without bIFN-\(\tau\) attained after treating cells with 0.5 ng/ml of bIFN-\(\tau\) was greater for concentration of PGF\(_{2\alpha}\) in the medium (60%) than for abundance of PGHS-2 mRNA (26.5%). The evidence that bIFN-\(\tau\) reduces PGF\(_{2\alpha}\) secretion through regulation of steady-state concentrations of PGHS-2 mRNA does not preclude other possible mechanisms. Inhibition of PGF\(_{2\alpha}\) secretion by bIFN-\(\tau\) is consistent with a previous report using similar concentrations of bIFN-\(\tau\) (50 ng/ml) and PDBu (100 ng/ml) [16]. Conversely, PGHS-2 mRNA concentrations were up-regulated by bIFN-\(\tau\) at high doses (1–20 \(\mu\)g/ml) [31]. This differential effect is related to the different doses of bIFN-\(\tau\), indicating that bIFN-\(\tau\) exerts a complex regulation of PGHS-2 gene expression and activity to modulate PGF\(_{2\alpha}\) production. Incubation for 24 h with EPA did not affect steady-state concentrations of PGHS-2 mRNA or intracellular abundance of PGHS-2 and PLA\(_2\) proteins. Similarly, concentrations of PGHS-2 were unaffected by AA and those of PLA\(_2\) only slightly decreased by DHA. Collectively, these findings suggest that the effects of EPA and AA on secretion of PGF\(_{2\alpha}\) are mediated by mechanisms that involve competition of precursors for processing by the PGHS enzymes and regulation of enzyme activity.

Experiment 9 demonstrated that incubation of BEND cells with EPA or with AA has opposite effects on secretion of PGF\(_{2\alpha}\), and supported the concept of competition for processing by the enzymes involved in prostanooid synthesis. The reduced secretion of PGF\(_{2\alpha}\) observed in cells incubated with EPA likely is the result of a shift of the PGHS pathway from synthesis of prostanooids from the 2 series to synthesis of prostanooids from the 3 series. The cyclooxygenase reaction has unique characteristics in its absolute requirement for hydroperoxide to stimulate its activity and in its suicide reaction that causes self-inactivation after approximately 1400 catalytic turnovers [32]. The PGHS enzymes differ in their ability to process different substrates. Eicosapentaenoic acid has been shown to be a poor substrate for PGHS-2, probably because the cyclooxygenase activity occurs in an inefficient manner, causing insufficient self-activation of the cyclooxygenase domain [8]. This mechanism of enzyme inhibition could be another factor causing reduced secretion of PGF\(_{2\alpha}\).

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**REFERENCES**

8. Kulmacz RJ, Pendleton RB, Lands WE. Interaction between peroxi-
10. Balapure AK, Ruxroad CE Jr, Kawada K, Watt DS, Fitz TA. Structural requirements for prostaglandin analog interaction with the ovine cor-
12. Meyer MD, Hansen PJ, Thatcher WW, Bovine trophoblast protein-1 complex alters endometrial protein and prostagl-
dandin secretion and induces an intracellular inhibitor of prostaglan-
13. Danet-Desnoyers G, Wettels C, Thatcher WW. Natural and recombi-
14. Xiao CW, Murphy BD, Sirios J, Goff AK. Down-regulation of oxy-
15. Binelli M, Guzeloglu A, Badinga L, Arnold DR, Sirios J, Hansen TR, Thatcher WW. Interferon-\(\tau\) modulates phorbol ester-induced produc-
tion of prostaglandin and expression of cyclooxygenase-2 and phospho-
lipase A\(_2\) from bovine endometrial cells. Biol Reprod 2000; 59:
293–297.
16. Stagg KL, Austin KJ, Johnson GA, Teixeira MG, Talbott CT, Doos-
17. Liu J, Antaya M, Boerboom D, Lussier JG, Silversides DW, Sirios J. The delayed activation of the prostaglandin G/H synthase-2 promoter in bovine granulosa cells is associated with down-regulation of trun-
18. Badinga L, Michel FJ, Fields MJ, Sharp DC, Simmen RCM. Pregnancy-
21. Austin KJ, King CP, Vierk JE, Sasser RG, Hansen TR. Pregnancy-


