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CLA and EPA inhibit LPS-induced prostaglandin release from bovine endometrial cells through an NF-κB-dependent signaling mechanism

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Abstract: Although their action in antagonizing arachidonic acid metabolism is a key antiinflammatory effect of conjugated linoleic acid (CLA) and omega-3 polyunsaturated fatty acids (*n*-3 PUFA), these molecules have other antiinflammatory effects that might occur upstream of altered eicosanoid production. We examined the effects of two CLA isomers (*c*9,*t*11 and *t*10,*c*12 isomers), *n*-3 PUFA (EPA and DHA), and κNF-κB inhibitor (ammonium pyrrolidine dithiocarbamate (PDTC)) on lipopolysaccharide (LPS)-induced secretion of prostaglandins (PGE_2 and $PGF_{2\alpha}$) by bovine endometrial (BEND) cells. LPS increased PGE_2 and $PGF_{2\alpha}$ concentrations in BEND cell-conditioned media in a concentration- and time-dependent manner. The *c*9,*t*11 CLA isomer and EPA decreased prostaglandin response to LPS. Addition of PDTC, alone or in combination with *c*9,*t*11 CLA or EPA, inhibited LPS-induced eicosanoid release into the culture medium. Results indicate that the *c*9,*t*11 CLA isomer and EPA inhibit the endometrial prostaglandin release in cattle and that these molecules may act through an NF-κB signaling mechanism.

Key words: Conjugated linoleic acid, eicosapentaenoic acid, prostaglandin, endometrium, cattle

1. Introduction

Infection of the endometrium with *Escherichia coli* causes uterine diseases and is generally associated with prolonged luteal phases of the ovarian cycle in early postpartum cows (1,2). In ruminants, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is luteolytic, whereas PGE_2 is luteotrophic (3). Under normal physiological conditions, $PGF_{2\alpha}$ is secreted by epithelial cells, whereas PGE_2 is secreted predominantly by stromal cells (2). However, when treated with lipopolysaccharide (LPS), both epithelial and stromal endometrial cells secrete considerably more PGE_2 than $PGF_{2\alpha}$ (2). This observation has led to the conclusion that bacterial LPS may induce an endocrine switch from $PGF_{2\alpha}$ to PGE_2 in bovine endometrium (2). Conjugated linoleic acid (CLA) is a collective term describing a mixture of positional and geometric dienoic isomers of linoleic acid (LA). Recent interest in CLA research stems from the well-documented anticarcinogenic, antiatherogenic, antidiabetic, and antiobesity properties of CLA in rodent models (4). One hypothesis of the mechanism of the antitumorigenic activity of CLA has been that this lipid alters the fatty acid composition of cell membrane phospholipids resulting in modulation of eicosanoids. In fact, a previous study in our laboratory demonstrated that CLA was a potent suppressor of phorbol ester-induced PGE_2 and $PGF_{2\alpha}$ in cultured bovine endometrial (BEND) cells (5). The fact that pretreatment with CLA did not affect prostaglandin H synthase-2 (PGHS-2) gene expression in cultured BEND cells suggested that, in this cell type, CLA modulation of prostaglandin (PG) biosynthesis may not involve the PGHS-2 gene expression.

Several studies have presented evidence that omega-3 polyunsaturated fatty acids (*n*-3 PUFA) decrease eicosanoid synthesis in the bovine uterus (6–8). Consistent with these in vivo studies, in vitro studies indicated that exogenous eicosapentaenoic (EPA) acid was a potent inhibitor of $PGF_{2\alpha}$ production by BEND cells (9,10). It has been postulated that supplemental *n*-3 PUFA may inhibit endometrial $PGF_{2\alpha}$ secretion by decreasing the availability of the precursor arachidonic acid, increasing the concentrations of fatty acids that compete with arachidonic acid for processing by PGHS-2, or inhibiting the PGHS-2 synthesis and or activity (7).

Nuclear factor κB (NF-κB) plays an important role in inducing a series of inflammatory genes, including PGHS-2, E-selectin, tumor necrosis factor-α, interleukin

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(IL)-1β, IL-6, inducible nitric oxide synthase, and matrix metalloproteinases in response to inflammatory stimuli (11). Nuclear factor κB exists as an inactive hetero-trimer in the cytosol of resting inflammatory cells (11). Upon stimulation, a signaling cascade phosphorylates IκB and induces NF-κB dissociation from the rest of the inactive trimer (11). The remaining transcription factor is translocated to the nucleus, where it activates the expression of target genes (11).

Because CLA and *n*-3 PUFA decrease inflammatory PG production in various experimental models (5,9,11), we hypothesized that these fatty acids may attenuate LPS-induced prostaglandin release by cultured BEND cells. The primary objective of this study was to examine the effects of exogenous CLA and *n*-3 PUFA on LPS-stimulated PGE_2 and PGF_{2a} production by BEND cells. A secondary objective was to determine if CLA and *n*-3 PUFA regulate endometrial PG production through an NF-κB-dependent signaling mechanism.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide from *E. coli* (serotype 0111:B4, purified by phenol extraction), pyrrolidine dithiocarbamate (PDTC), and specific reagents for cell culture were obtained from Sigma Aldrich (St. Louis, MO, USA). The CLA isomers (*c*9,*t*11 CLA, purity ≥96% and *t*10,*c*12 CLA, purity ≥98%), EPA, and docosahexaenoic acid (DHA) were from Cayman Chemical Co. (Ann Arbor, MI, USA). Enzyme-linked immunosorbent assay (ELISA) kits for bovine PGE_2 and PGF_{2a} were from Enzo Life Sciences (Plymouth Meeting, PA, USA). Immortalized BEND cells were kindly provided by Dr Todd Hansen (Colorado State University, Fort Collins, CO, USA).

2.2. Lipopolysaccharide stimulation of PGE_2 and PGF_{2a} production

To evaluate the effect of LPS on PGE_2 and PGF_{2a} production, subconfluent BEND cells (n = 3 passages for each treatment) were incubated with increasing concentrations of LPS (0, 0.1, 1.0, and 10.0 μg/mL) for 24 h or with 1 μg/mL LPS for increasing times (0, 4, 12, and 24 h). After LPS challenge, samples of cell-conditioned media (500 μL) were collected from each well and stored at –80 °C for subsequent PGE_2 and PGF_{2a} determination. Stimulation with 1 μg/mL LPS for 24 h elicited the optimal physiological effects and, therefore, these conditions were used for all subsequent experiments.

2.3. Fatty acid treatment of BEND cells

To examine CLA and *n*-3 PUFA modulation of LPS-induced PGE_2 and PGF_{2a} production, replicate samples of subconfluent BEND cells were pretreated with the fatty acids (100 μM) for 24 h and then challenged with LPS (1 μg/mL) for an additional 24 h. Fatty acids were complexed with bovine serum albumin (BSA; 1:2 ratio) for 2 h in a 37 °C water bath prior to addition to the cultures. The concentrations of fatty acids and the duration of fatty acid treatment (24 h) were based on previous studies in our laboratory, which indicated that these concentrations of PUFA elicited maximal physiological effects at 24 h of treatment of BEND cells (5,9). Cultures were then challenged with LPS (1 μg/mL) for an additional 24 h. After LPS treatment, samples of cell-conditioned media (500 μL) were collected and stored at –80 °C for subsequent PG assays.

2.4. Inhibition of eicosanoid release by NF-κB inhibitor

To evaluate NF-kB involvement in endometrial PG production, PDTC (50 μM) was added to appropriate cultures 30 min before addition of LPS (12). A set of wells received no treatment and served as the experimental control. All culture wells received the same amounts of treatment vehicles. After LPS challenge, samples of cell-conditioned media (500 μL) were collected and stored at –80 °C for subsequent PG determination.

2.5. Prostaglandin assay

Concentrations of PGE_2 and PGF_{2a} in culture media were determined by ELISA following the manufacturer's instructions. The least detectable concentrations were 13.4 and 6.5 pg/mL for PGE_2 and PGF_{2a}, respectively. Intra- and interassay coefficients of variation for both analytes were less than 10%.

2.6. Statistical analyses

Prostaglandin response to LPS was analyzed using the general linear models procedure in the SAS software package (SAS Institute Inc., Cary, NC, USA). In preliminary experiments, the statistical models included the effects of dose or time of incubation. For fatty acid experiments, the statistical models included the fixed effects of fatty acids. Wells, nested within treatments, were considered as experimental units, and therefore the well variance was used to test all treatment effects. Treatment means were further separated using preplanned orthogonal contrasts. Differences were considered to be significant at $P < 0.05$. Data are presented as least squares means ± standard error of the mean (SEM).

3. Results

3.1. Dose and time dependence of prostaglandin response to LPS

Addition of LPS (1 μg/mL) to the culture medium increased ($P < 0.01$) PGE_2 and PGF_{2a} release in a concentration- and time-dependent manner (Figures 1A, 1B, 2A, and 2B). The optimal stimulation of PGE_2 was achieved with 1 μg/mL LPS at 24 h (Figures 1A and 2A). Concentrations of PGF_{2a} in the culture medium increased linearly between 0 and 10 μg/mL LPS (Figures 1B and 2B). On average, endometrial

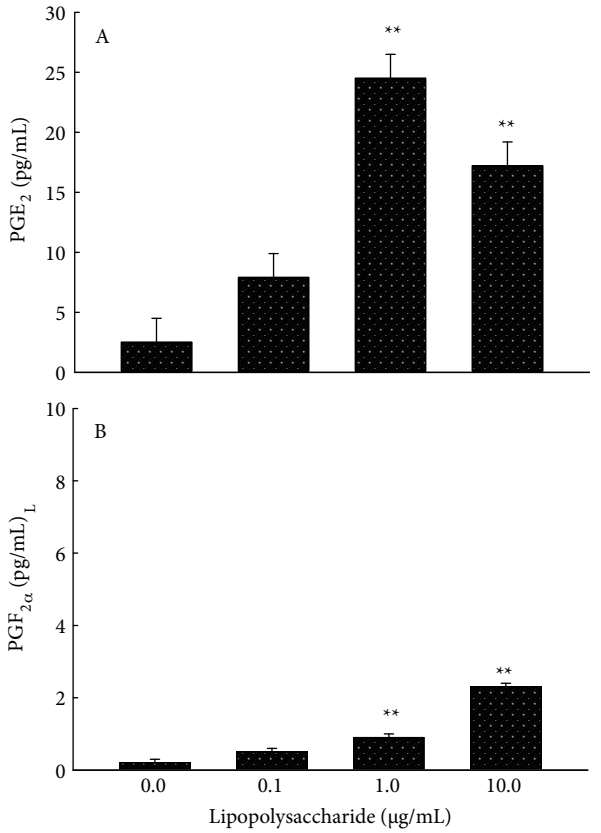

Figure 1. Lipopolysaccharide (LPS) stimulated prostaglandin E_2 (A) and F_{2a} (B) in a concentration-dependent manner. Subconfluent bovine endometrial (BEND) cells were incubated with increasing concentrations of LPS (0–10 μg/mL) for 24 h. Concentrations of PGE_2 and PGF_{2a} in cell-conditioned media were measured by specific bovine ELISA as described in Section 2. Asterisks indicate that the specified means are different from the control mean at $P < 0.01$.

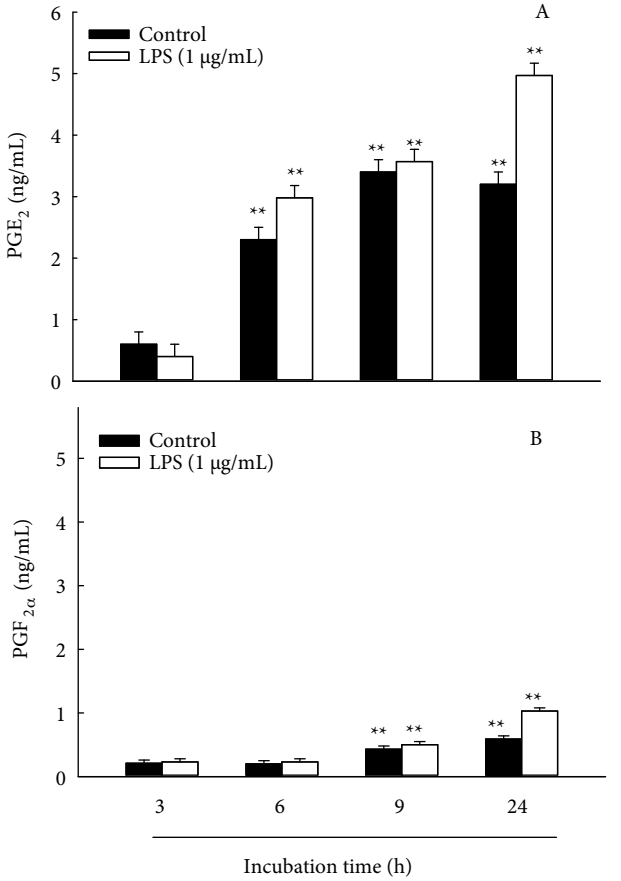

Figure 2. Lipopolysaccharide (LPS) stimulated prostaglandin E_2 (A) and F_{2a} (B) in a time-dependent manner. Subconfluent bovine endometrial (BEND) cells were stimulated with 1 μg/mL LPS for increasing times (3–24 h). Concentrations of PGE_2 and PGF_{2a} in cell-conditioned media were measured by specific bovine ELISA as described in Section 2. Asterisks indicate that the specified means are different from the control mean at $P < 0.01$.

cells treated with LPS produced significantly ($P < 0.01$) more PGE_2 than PGF_{2a} (Figures 1 and 2). The mean PGE_2/PGF_{2a} ratio was 16.

3.2. Fatty acid modulation of prostaglandin production

Pretreatment of BEND cells with *c*9,*t*11 CLA isomer (100 μM) abrogated ($P < 0.01$) PGE_2 and PGF_{2a} response to LPS (Figure 3). The *t*10,*c*12 CLA isomer (100 μM) was less effective at reducing LPS-induced eicosanoid release by BEND cells (Figure 3). Pretreatment of BEND cells with EPA (100 μM) decreased ($P < 0.01$) PGE_2 and PGF_{2a} responses to LPS (Figure 4). Unlike EPA, priming of BEND cells with DHA had minimal effects on in vitro PG response to LPS (Figure 4).

3.3. Involvement of NF-κB in endometrial PG production

Pretreatment of BEND cells with PDTC (50 μM) completely abolished ($P < 0.01$) LPS-induced release of PG into the culture media (Figures 5 and 6).

4. Discussion

Lipopolysaccharide, a surface component of gram-negative bacteria released upon host infection, is one of the strongest stimuli of PGE_2 production in uterine endometrial cells (2). Consistent with previous studies (2,13,14), LPS induced PGE_2 and $PGF_{2\alpha}$ production in a concentration- and time-dependent manner. Endometrial cells treated with LPS secreted considerably more PGE_2 than $PGF_{2\alpha}$ (Figures 1 and 2). The cellular and molecular mechanisms for the preferential synthesis of PGE_2 over $PGF_{2\alpha}$ are complex and may involve coordinated upregulation of PGHS-2 and microsomal PGE synthase 1 transcription by the endotoxin (15,16). In support of this hypothesis, large amounts of PGE_2 are produced by macrophages during the inflammatory process, due to increased expression of both PGHS-2 and mPGES-1

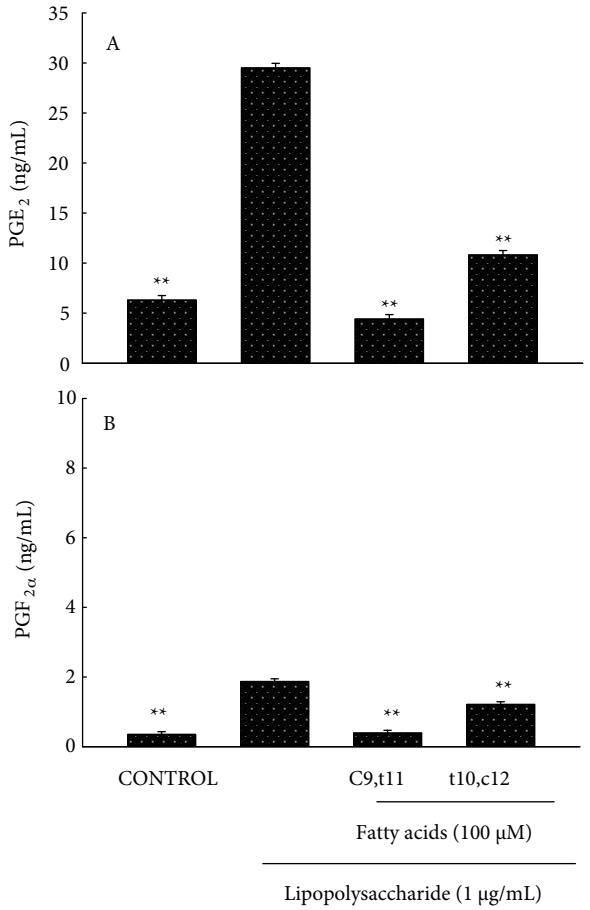

Figure 3. *c*9,*t*11 conjugated linoleic acid (CLA) completely abolished lipopolysaccharide (LPS)-stimulated prostaglandin E_2 (A) and $F_{2\alpha}$ (B) production by cultured bovine endometrial (BEND) cells. Subconfluent BEND cells were pretreated with CLA isomers (100 μM) for 24 h and then challenged with LPS (1 μg/mL) for an additional 24 h. Concentrations of PGE_2 and PGF_{2a} in cell-conditioned media were measured by specific bovine ELISA as described in Section 2. Asterisks indicate that the specified means are different from the control and EPA means at $P < 0.01$.

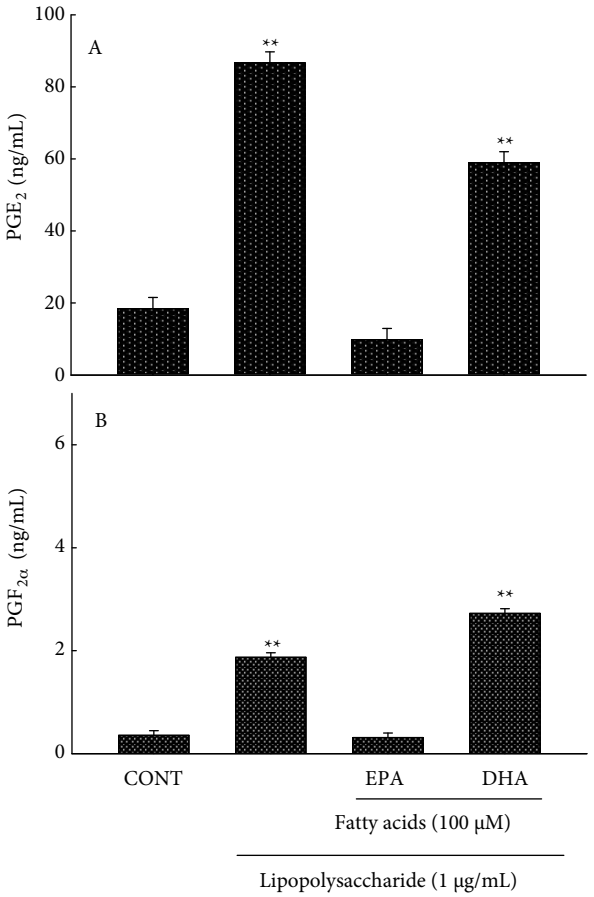

Figure 4. Eicosapentaenoic acid (EPA) decreased lipopolysaccharide (LPS)-stimulated prostaglandin E_2 (A) and $F_{2\alpha}$ (B) production by cultured bovine endometrial (BEND) cells. Subconfluent BEND cells were pretreated with eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) (100 μM) for 24 h and then challenged with LPS (1 μg/mL) for an additional 24 h. Concentrations of PGE_2 and PGF_{2a} in cell-conditioned media were measured by specific bovine ELISA as described in Section 2. Asterisks indicate that the specified means are different from the control and EPA means at $P < 0.01$.

(17,18). However, these observations do not rule out the possibility that LPS may affect upstream enzymatic steps leading to endometrial PG biosynthesis.

The synthesis of eicosanoids starts with the release of arachidonic acid from membrane stores by PLA2 enzymes, and there are at least two groups of PLA2 enzymes (PLA2G4C and PLA2G6) involved in bovine endometrial function (19,20). Endometrial cells overexpressing PL2G4C produced more PGE_2 than $PGF_{2\alpha}$ and interferon-tau stimulated PGE_2 production (19). This was in contrast to oxytocin induction of PLA2G6 leading to accumulation of $PGF_{2\alpha}$ (2). These observations collectively indicate that endometrial synthesis of PGE_2 and $PGF_{2\alpha}$ may be mediated through distinct PLA2 enzymes (19,20).

Priming of BEND cells with c9,*t*11 CLA isomer decreased LPS-induced release of PG. This effect was isomer-specific because the *t*10,*c*12 CLA isomer was less effective at reducing LPS-stimulated eicosanoid production by BEND cells. These results extend previous observations that supplemental CLA reduces PG production in mammalian models (5,21). The mechanism underlying CLA inhibition of PG synthesis is complex and may involve more than one pathway. Banni et al. (22) reported that CLA supplementation of rat mammary tissue increased the accumulation of CLA metabolites at the expense of

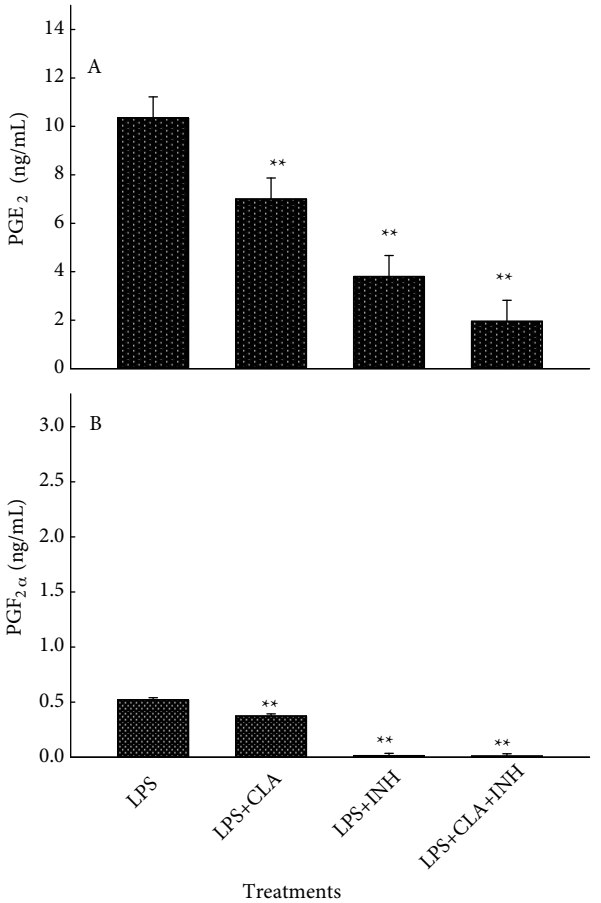

Figure 5. Ammonium pyrrolidine dithiocarbamate (PDTC) interacted with conjugated linoleic acid (CLA) to decrease lipopolysaccharide (LPS)-induced prostaglandin E_2 (A) and F_{2a} (B) in cultured bovine endometrial (BEND) cells. Subconfluent BEND cells were pretreated with *c*9,*t*11 conjugated linoleic acid (CLA) isomer (100 μM) or PDTC (50 μM) for 24 and 0.5 h, respectively, before addition of LPS. The cells were then challenged with LPS (1 μg/mL) for 24 h. Concentrations of PGE_2 and PGF_{2a} in cell-conditioned media were measured by specific bovine ELISA as described in Section 2. Asterisks indicate that the specified means are different from the LPS mean at $P < 0.01$.

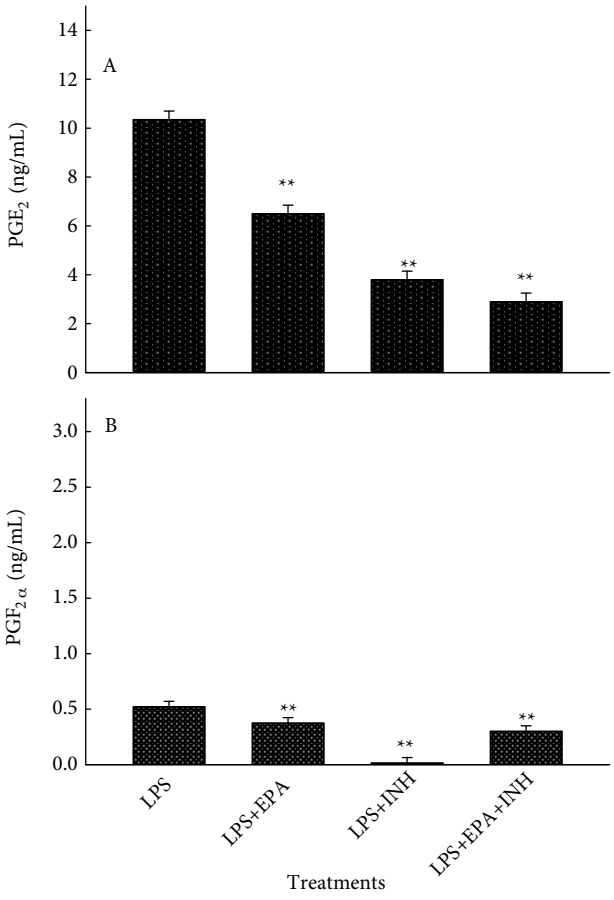

Figure 6. Ammonium pyrrolidine dithiocarbamate (PDTC) interacted with eicosapentaenoic acid (EPA) to decrease lipopolysaccharide (LPS)-induced prostaglandin E_2 (A) and F_{2a} (B) in cultured bovine endometrial (BEND) cells. Subconfluent BEND cells were pretreated with EPA (100 μM) or PDTC (50 μM) for 24 and 0.5 h, respectively, before addition of LPS. The cells were then challenged with LPS (1 μg/mL) for 24 h. Concentrations of PGE_2 and PGF_{2a} in cell-conditioned media were measured by specific bovine ELISA as described in Section 2. Asterisks indicate that the specified means were different from the LPS mean at $P < 0.01$.

LA metabolites in the tissue. Because CLA metabolites are potent inhibitors of PGHS-2 and lipoxygenase enzymes (23), it has been suggested that supplemental CLA may decrease PG production, in part, by inhibiting PGHS-2 enzyme activity in cultured mammalian cells. Additionally, CLA may have effects at the level of gene expression. In fact, Iwakiri et al. (24) showed that CLA 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 NF-κB. It is unlikely that this mechanism of gene repression is functional in BEND cells, because CLA failed to attenuate PGHS-2 mRNA response in phorbol dibutyrate-stimulated BEND cells (5).

Consistent with previous studies (9,10), pretreatment of BEND cells with EPA attenuated PGE_2 and $PGF_{2\alpha}$ response to LPS. In contrast, DHA, another long chain *n*-3 PUFA, had negligible effects on LPS-induced PG production by cultured BEND cells. The exact physiological or molecular mechanisms by which EPA alters the release of PGE_2 and $PGF_{2\alpha}$ in response to LPS are not fully understood. Polyunsaturated fatty acids are key structural and functional components of the phospholipids in cell membranes (11). The phospholipids of cells involved in

inflammation generally contain more arachidonic acid than *n*-3 PUFA (25,26). However, including fish oil in the diet of experimental animals (7,25) or healthy human volunteers (27,28) results in the increased accumulation of EPA and DHA in these cells and decreased arachidonic acid content in the phospholipid pool. Because both EPA and arachidonic acid are substrates for the PGHS enzymes, increased availability of EPA in the membrane likely leads to increased synthesis of prostanoids of the 3 series at the expense of prostanoids of the 2 series (10,28). Additionally, EPA may reduce the activity or expression of PLA2 and/or PGHS genes (29), which would make the PLA2 and PGHS enzymes less available and reduce PGE_2 and $PGF_{2\alpha}$ synthesis.

NF-κB is normally confined in the cytoplasm through its association with IκB. When cells are activated by cytokines, oxidants, and LPS, the IκB is rapidly phosphorylated and degraded to free the NF-κB. The transcription factor then migrates to the nucleus, where it binds to the cognate DNA binding site and activates inflammatory gene transcription (11). Thus, in addition to affecting arachidonic acid metabolism and PGHS-2 activity (30), CLA and EPA may decrease LPS-induced eicosanoid release by preventing nuclear translocation of NF-κB. Support for this hypothesis was provided by the finding that PDTC, an NF-κB inhibitor, completely abrogated LPS-induced eicosanoid release in cultured BEND cells.

In conclusion, results of this study provide further evidence that CLA and EPA are potent suppressors of endometrial PG production and that these molecules may act through an NF-κB signaling pathway.

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