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# Peroxisome proliferator-activated receptor (PPAR) expression in cultured bovine endometrial cells and response to omega-3 fatty acid, growth hormone and agonist stimulation in relation to series 2 prostaglandin production

Leslie A. MacLaren<sup>a</sup>, Aydin Guzeloglu<sup>b</sup>,  
Frank Michel<sup>c</sup>, William W. Thatcher<sup>c,\*</sup>

<sup>a</sup> Department of Plant and Animal Sciences, Nova Scotia Agricultural College,  
Truro, Nova Scotia, Canada B2N 5E3

<sup>b</sup> Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine,  
Selcuk University, Konya 42075, Turkey

<sup>c</sup> Department of Animal Sciences, 459 Shealy Drive, University of Florida,  
Gainesville, Florida FL 32611, USA

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## Abstract

The peroxisome proliferator-activated receptors (PPARs) are a family of nuclear transcription factors thought to act as receptors for polyunsaturated fatty acids and to reduce production of series 2 prostaglandins (PG). The objectives of the current study were to characterize PPAR expression and the prostaglandin synthetic activity of cultured bovine endometrial cells in response to known PPAR ligands, as well as to key stimulators and inhibitors of series 2 prostaglandin secretion. PPAR $\alpha$  and PPAR $\delta$ , but not PPAR $\gamma$ , mRNAs are expressed in the BEND cell line regardless of treatment. Under resting conditions, PPAR $\alpha$  mRNA levels increase in response to growth hormone ( $P < 0.05$ ). In cells stimulated with PdBu, growth hormone depresses PPAR $\alpha$  mRNA levels, regardless of whether cells also are treated with IFN $\tau$ . In contrast, PPAR $\delta$  mRNA levels are increased by exposure to PdBu, eicosapentanoic acid and IFN $\tau$ , and these effects are additive. PPAR mRNA levels are not predictive of prostaglandin accumulation. Agonist activation of PPAR $\alpha$ , PPAR $\delta$  or PPAR $\gamma$  augments PdBu-induced

\* Corresponding author. Tel.: +1 352 392 5590; fax: +1 352 392 5595.  
E-mail address: [thatcher@animal.ufl.edu](mailto:thatcher@animal.ufl.edu) (W.W. Thatcher).

increases in prostaglandin H synthase-2 mRNA and media accumulation of prostaglandins F<sub>2α</sub> and E<sub>2</sub>. Treatment with the PPARα/δ agonist carbaprostacyclin, but not the PPARα agonist Wy14643 or PPARγ agonist ciglitazone, completely reverses the IFNτ suppression of prostaglandin synthesis. In conclusion, PPARα and PPARδ function in the response of bovine endometrium to growth hormone and long chain omega-3 polyunsaturated fatty acids.

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*Keywords:* Pregnancy; Female reproductive tract; Polyunsaturated fatty acid; Peroxisome proliferator-activated receptor; Prostaglandin

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## 1. Introduction

The peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors activated by selected long chain fatty acids, eicosanoids and peroxisome proliferators. There are three subtypes of PPAR (i.e., α, δ and γ) that upon ligand binding, heterodimerize with the retinoid receptor RXR and interact with specific PPAR response elements in the promoter region of target genes to affect transcription. Regulation of promoter function is complex, since there is tissue specific expression of the PPAR and RXR subtypes, competition for the RXR binding partner, and differences in binding affinity among the PPAR subtypes and among the RXR subtypes [1]. PPAR activation may be ligand dependent or independent, and there is also cross-talk with other nuclear receptors and their response elements, as well as several transcription factors [1,2]. The PPARs are best known for their roles in lipid metabolism, but they are also involved in development, nervous tissue, lung, kidney and cardiac functions, epidermal maturation and reproduction in several animal models [1,3].

A distinctive feature of ruminant endometrium is its role in control of the estrous cycle. If the female is not pregnant, at about day 16 postestrus the endometrial epithelial cells differentiate in response to oxytocin receptor stimulation and secrete large amounts of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) in a pulsatile manner [4]. This PGF<sub>2α</sub> is delivered to the ovarian arterial blood from the uterine vein by a countercurrent mechanism, and causes luteolysis and subsequent return to estrus at about day 21. During pregnancy, the trophoblast of the elongating embryo produces interferon-tau (IFNτ), which suppresses oxytocin receptor upregulation and pulsatile PGF<sub>2α</sub> secretion. Supplementation of dairy cow diets with long chain omega-3 polyunsaturated fatty acids (PUFA) such as those found in flax or fish oil improves reproductive performance, likely by decreasing uterine PGF<sub>2α</sub> production and increasing embryo survival [5,6]. Our recent studies with a bovine cell line derived from endometrial epithelial cells showed that omega-3 long chain PUFA added to culture medium depress PGF<sub>2α</sub> secretion [7], and others have found similar results using cultured human decidual cell lines [8]. The mechanism for this inhibition is not known, but long chain omega-3 PUFA are known to affect eicosanoid signaling in a variety of ways: they reduce the efficiency of the prostaglandin H synthase (also known as cyclooxygenase) enzymes which regulate prostanoid synthesis, increase eicosanoid catabolism and favor the production of less biologically active products, including the production of series 1 and 3 prostaglandins at the expense of series 2 prostaglandins [9,10]. There is evidence that

some of these effects may be mediated through PPAR activation, since the omega-3 PUFA are ligands for all three PPARs [9,11]. In murine endometrium, a strong link has been made between prostaglandin H synthase-2 (PGHS-2) activity, prostaglandin I<sub>2</sub> production (PGI<sub>2</sub>) and activation of PPAR $\delta$  [12]. Induction of PGHS-2 expression by PPAR activation is observed in other model systems, and a putative PPRE is present in the human PGHS-2 promoter [13]. In airway smooth muscle cells, this response element is stimulated by PPAR $\gamma$  agonists [14].

Collectively, these observations indicate that there may be a role for one or more PPARs in bovine endometrial prostaglandin production. The objective of this study was to characterize PPAR expression and the prostaglandin synthetic activity of cultured bovine endometrial cells in response to known PPAR ligands, as well as to key stimulators and inhibitors of series 2 prostaglandin secretion.

## 2. Materials and methods

### 2.1. Materials

Recombinant bovine IFN $\tau$  ( $1.08 \times 10^7$  units of antiviral activity per mg) and recombinant bovine growth hormone (GH) were generous gifts from Dr. R. Michael Roberts (University of Missouri, Columbia, MO) and Monsanto Co. (St. Louis, MO), respectively. Polystyrene tissue culture dishes (100  $\times$  20) were from Corning (Corning Glass Works, Corning, NY). Acrylamide, *N,N'*-methylenebisacrylamide, SDS, and Nonidet-P40 were purchased from BDH Laboratory Supplies (Poole, UK). Eicosapentanoic acid was purchased from Cayman Chemicals (Ann Arbor, MI). The PPAR $\alpha$  agonist Wy14643 and the PPAR $\gamma$  agonist ciglitazone were purchased from Calbiochem (EMD Biosciences Inc., San Diego, CA). Carbaprostacyclin, Coomassie brilliant blue, bromophenol blue,  $\beta$ -mercaptoethanol, NaOH, Tris, Tris-HCl, TEMED, ammonium persulfate, formaldehyde, acetic acid, Tween 20, NaCl, EDTA, NaF, glycerol, glycine, and methanol were from Fisher Scientific (Pittsburgh, PA). The phorbol 12,13 dibutyrate, Hams F-12 culture medium, antibiotic-antimycotic solution, insulin, D-valine and other laboratory chemicals were from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Isotopically labeled [5, 6, 8, 11, 12, 14, 15-<sup>3</sup>H]-PGF<sub>2 $\alpha$</sub>  and -PGE<sub>2</sub>, and the nitrocellulose membranes (Hybond-ECL) were from Amersham Corporation (Arlington Heights, IL). X-ray film was from Eastman Kodak Co. (X-Omat Blue XB-1; Rochester, NY).

### 2.2. BEND cell culture and treatments

The isolation, characterization and culture of the BEND cell line is described elsewhere ([15]; American Type Culture Collection number CRL-2398; ATCC, 10801 University Boulevard, Manassas, VA 20110-2209), but briefly this cytokeratin-positive uterine cell line derived from a diestrus cow maintains an epithelial cell phenotype. It produces PGF<sub>2 $\alpha$</sub>  in response to stimulation of protein kinase C, the downstream mediator of oxytocin receptor activation *in vivo*, by the phorbol ester PDBu. The BEND cells were cultured in 100-mm dishes ( $1 \times 10^4$  cells/cm<sup>2</sup>) in culture medium (40% Hams F-12, 40% MEM, 1% antibiotic/

antimycotic, 0.2 U/ml insulin, 34.3 mg/l D-valine, 10% fetal bovine serum, and 10% horse serum) at 37 °C under a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> until 90% confluence. The cells were washed in serum-free medium, then cultured for an additional 24 h in serum-free medium. At this time (designated 0h), cultures were either not treated (Basal) or treated with 100 ng/ml phorbol 12,13 dibutyrate (PdBu) to stimulate prostaglandin synthesis, or PdBu plus 50 ng/ml IFN $\tau$  (PdBu-IFN), a treatment known to suppress prostaglandin synthesis [4]. Cells and media were harvested 3 h post-treatment, a time known to be sufficient for demonstration of significant treatment effects [4].

The effects of the omega-3 polyunsaturated fatty acid eicosapentanoic acid (EPA) were examined by supplementing medium beginning at –24 h with 100  $\mu$ M EPA, prepared as described by Mattos et al. [7]. Cultures not receiving EPA received BSA carrier at the same concentrations as treated cultures. For growth hormone treatments, 50 ng/ml recombinant bovine growth hormone (GH) were added to the culture medium 6 h precollection (–3 h) as described by Badinga et al. [16]. Agonists used to stimulate PPARs were added in culture medium at –24 h, and included 10  $\mu$ M ciglitizone, a PPAR $\gamma$ -specific activator, 10  $\mu$ M Wy14643, a PPAR $\alpha$ -specific activator, and 1  $\mu$ M carbaprostacyclin, an analogue of prostaglandin I<sub>2</sub> that is known to activate both PPAR $\alpha$  and PPAR $\delta$ .

### 2.3. Extraction of RNA and Northern blots

Total RNA was isolated from endometrial cells using Trizol<sup>®</sup> according to the manufacturer's recommendations (Invitrogen Corporation, Carlsbad, CA), then quantified by spectrophotometry. Intron-spanning primers were designed to amplify cDNA from mRNA transcribed by the bovine PPAR $\alpha$ , PPAR $\delta$  and PPAR $\gamma$  genes (Table 1). For PPAR $\gamma$ , the primers recognized a sequence common to both PPAR $\gamma$ 1 and PPAR $\gamma$ 2 gene products. Total RNA (1  $\mu$ g) from kidney (i.e., for PPAR $\delta$  and PPAR $\alpha$ ) and adipose (i.e., for PPAR $\gamma$ ) was reversed transcribed with AMV reverse transcriptase using a commercial cDNA synthesis kit (Invitrogen). The polymerase chain reaction was carried out using 100 ng of forward and reverse primer and 1  $\mu$ l of the cDNA reaction product in a 50  $\mu$ l reaction mix containing Taq polymerase (Boehringer-Mannheim) for 40 cycles in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf Scientific Inc., Westborg, NY). The PCR products were subcloned into the TOPO<sup>®</sup> plasmid vector (Invitrogen). The nucleotide sequences of the generated clones were determined at the nucleotide sequencing facility of DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research of the University of Florida, and compared with published PPAR mRNA sequences.

Table 1  
Primer design for cDNA amplification of bovine PPAR mRNA

Amplicon	Forward primer	Reverse primer	Fragment size	GenBank accession number
PPAR $\alpha$	5'-aataacgcgattcgttttg-3'	5'-tccatgctggtgatgagaaa-3'	233	AF229356
PPAR $\delta$	5'-cactctactgctgaccaa-3'	5'-tccatgctggtgatgagaaa-3'	216	AF229357
PPAR $\gamma$	5'-ccctggcaagcatttgat-3'	5'-actgacaccctggaagatg-3'	222	Y12419/Y12420

The relative levels of PPAR mRNAs were assessed by Northern blotting. For Northern blots, 30 µg of total RNA were electrophoresed in 1% agarose–formaldehyde gels and blotted to nylon membrane. Membrane bound RNA was crosslinked by UV radiation and baked at 80 °C for 1 h. The blots were prehybridized with ULTRAhyb® (Ambion Inc., Austin, TX) for 1 h at 42 °C, then hybridized with random primed-<sup>32</sup>P-labelled cDNA probes for either PPAR $\alpha$ , PPAR $\delta$  or PPAR $\gamma$  overnight at 42 °C. The next day, the blots were washed in 2 × SSC/0.1% SDS and twice in 0.1 × SSC/0.1% SDS for 20 min each at 42 °C, then exposed to X-ray film. Blots were stripped with 1% SDS for 20 min, then reprobed with cDNA probes specific for bovine prostaglandin H synthase-2 (PGHS-2; gift from Dr. J. Sirois, University of Montreal, Que., Canada) and bovine glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA. The latter was used as a housekeeping control for RNA loading. Densitometry (Alpha Imager 2000, Alpha Innotech Corporation, San Leandro, CA) was used to compare pixel intensity of PPAR, PGHS-2 and GAPDH transcripts within each experiment.

#### 2.4. Radioimmunoassay for prostaglandins

Concentrations of PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> were measured in culture media 3 h post-PdBu treatment by direct RIA as described by Danet-Desnoyers et al. [17] and Gross et al. [18], respectively. Both antisera were characterized by Dubois and Bazer [19]. The anti-PGF<sub>2 $\alpha$</sub>  antiserum was diluted 1:5000 and the anti-PGE<sub>2</sub> antiserum was diluted 1:1000 in Tris buffer. When assaying 25 µl of media, sensitivities of the assays were 5 pg for PGF<sub>2 $\alpha$</sub>  and 50 pg for PGE<sub>2</sub>. Cross-reactivities to prostaglandin I<sub>2</sub> were determined to be negligible for each assay (data not shown).

#### 2.5. Data expression and statistical analysis

Effects of treatment on PPAR RNA levels and prostaglandin accumulation within experiment were determined by analysis of variance using the general linear models procedure in SAS<sup>TM</sup> (Statistical Analysis Software Version 8, SAS Institute Inc., Cary, NC). For RNA analyses, pixel intensity of the GAPDH transcript (nested within experiment if appropriate) was included as a covariate in the model to adjust for RNA loading. Least squares treatment means were compared using Tukey's test for multiple comparison of means. The selected  $\alpha$  error rate was  $P \leq 0.05$ . The effects of treatments on relative GAPDH mRNA levels were tested and confirmed to be non-significant for all datasets ( $P > 0.10$ ).

### 3. Results

#### 3.1. Northern blot identification of PPAR mRNA

Northern blot analysis of BEND cell extracts revealed expression of a single transcript for bovine PPAR $\alpha$  of approximately 9 kb in length and a single transcript for bovine PPAR $\delta$  of approximately 3.8 kb (Fig. 1). These transcript sizes correspond to the previously observed sizes of the bovine and human PPAR $\alpha$  and PPAR $\delta$  mRNAs (H. Sundvolt, Agricultural

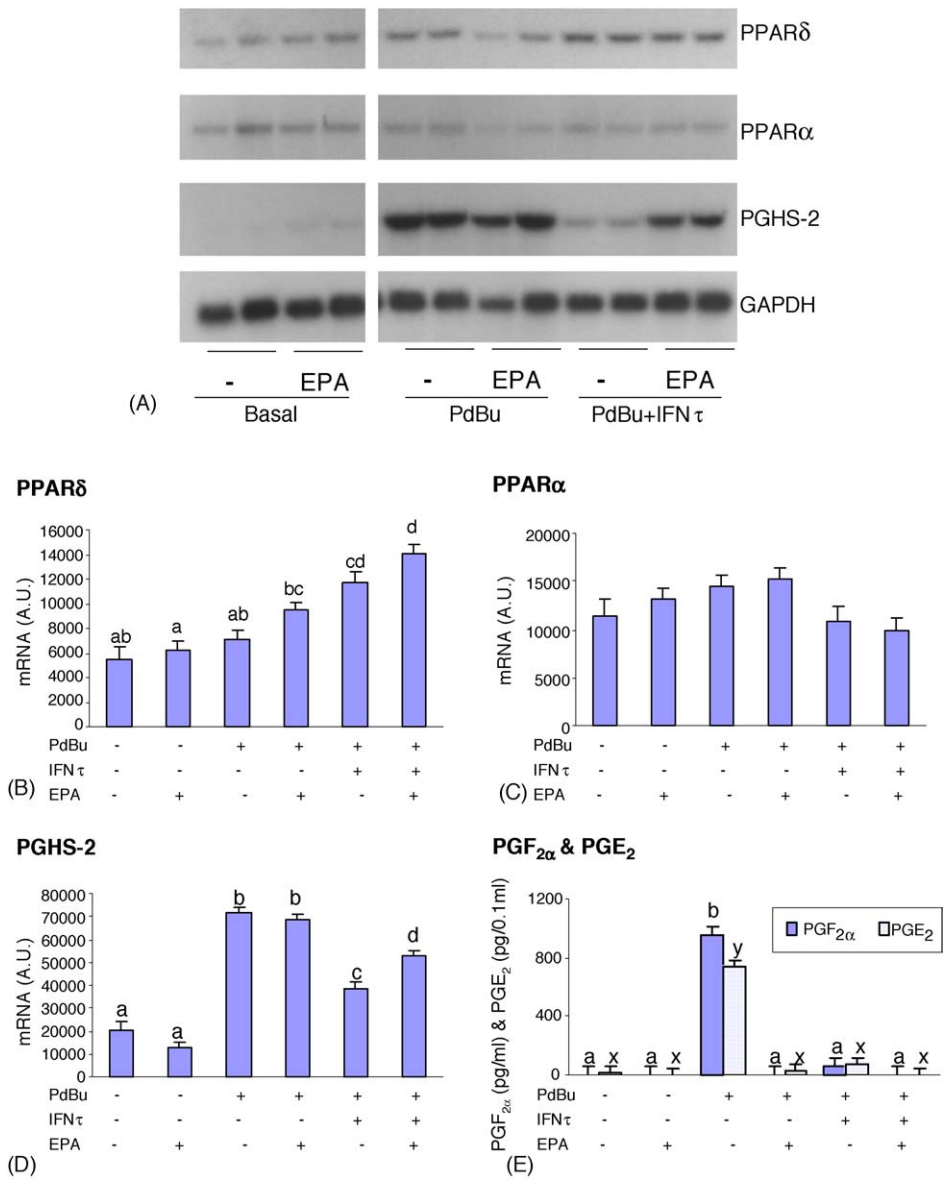


Fig. 1. Effects of treatment with 100 μM EPA and/or 50 ng/ml IFNτ on BEND cells stimulated with 100 ng/ml PdBu for 3 h. (A) Total RNA was isolated from BEND cells and subjected to Northern blot analysis using specific bovine PPARδ, PPARα, PGHS-2 and GAPDH cDNA probes. GAPDH mRNA was used as a loading control. (B–D) Least squares mean ± S.E. of indicated mRNA levels in three experiments each with duplicate samples after adjustment for loading. Treatments with different letters were different (*P* < 0.05). (E) Accumulation of PGF<sub>2α</sub> (pg/ml) and PGE<sub>2</sub> (pg/0.1 ml) in BEND cell supernatants was measured using RIA 3 h after PdBu treatment. Prostaglandin values are LS mean ± S.E. of two experiments each performed in duplicate. Significant (*P* < 0.05) differences among treatments are indicated by different letters within prostaglandin type.

Table 2

Effect of IFN $\tau$  and EPA on least squares mean  $\pm$  S.E. of PPAR mRNA levels in BEND cells in the absence of phorbol ester stimulation

mRNA	Treatment				Significance of <i>F</i>
	Basal	EPA	IFN $\tau$	EPA + IFN $\tau$	
PPAR $\delta$	17059 $\pm$ 1665	17876 $\pm$ 2269	15185 $\pm$ 2332	15492 $\pm$ 2066	<i>P</i> > 0.05
PPAR $\alpha$	24603 $\pm$ 2279	23770 $\pm$ 3105	16815 $\pm$ 3191	17543 $\pm$ 2827	<i>P</i> > 0.05

University of Norway, personal communication; [20,21]). Regardless of treatment, PPAR $\gamma$  mRNA was not detected at significant levels in BEND cells, although samples of ovarian or adipose mRNA indicated that our probe recognized two transcripts, approximately 2.1 and 2.3 kb (data not shown).

### 3.2. PdBu and IFN $\tau$ effects on PPAR and PGHS-2 mRNA expression, and prostaglandin synthesis

In the absence of PdBu, neither PPAR $\delta$  nor PPAR $\alpha$  mRNA levels were affected by treatment with IFN $\tau$  (Table 2). Treatment of BEND cells with 100 ng/ml PdBu for 3 h increased steady-state levels of PPAR $\delta$  mRNA (Fig. 1), resulting in an increase in the least squares mean for basal PPAR mRNA expression in arbitrary units from 5469  $\pm$  912 to 8034  $\pm$  666 in PdBu-treated cells (*P* < 0.05; means include treatments with and without EPA). Addition of IFN $\tau$  to PdBu-treated cultures further increased the least squares mean of samples with and without EPA to 11687  $\pm$  900 arbitrary units (*P* < 0.05). Neither PdBu nor PdBu + IFN $\tau$  treatment consistently affected PPAR $\alpha$  mRNA levels (*P* > 0.10). In contrast, PdBu consistently increased PGHS-2 mRNA levels (*P* < 0.05), and PdBu-IFN $\tau$  partially reversed this effect (*P* < 0.05).

Accumulation of PGF $_{2\alpha}$  and PGE $_2$  in culture media followed similar patterns in response to the experimental treatments, although PGE $_2$  levels were approximately 10-fold higher. Levels of both prostaglandins were below detection limits of the assays without PdBu treatment, regardless of whether EPA and/or GH were present (Figs. 1 and 2). Three hour treatment with PdBu consistently stimulated accumulation of both prostaglandins (*P* < 0.01). Combining PdBu stimulation with IFN $\tau$  suppressed prostaglandin levels (*P* < 0.01) such that they were not different from those observed in unstimulated cells (Fig. 1).

### 3.3. Effects of EPA and GH on PPAR and PGHS-2 mRNA expression, and prostaglandin synthesis

Although addition of EPA at –24 h did not influence basal PPAR $\delta$  expression consistently (*P* > 0.05), orthogonal contrasts indicated that EPA increased PPAR $\delta$  mRNA levels in PdBu- and PdBu-IFN $\tau$ -treated cells relative to controls (*P* < 0.05; Fig. 1A and B). Levels of PPAR $\alpha$  mRNA were not affected by EPA (*P* > 0.05; Fig. 1C). Pretreatment of PdBu-treated BEND cells with EPA partially reversed the IFN $\tau$  suppression of PGHS-2 mRNA (*P* < 0.05; Fig. 1D).

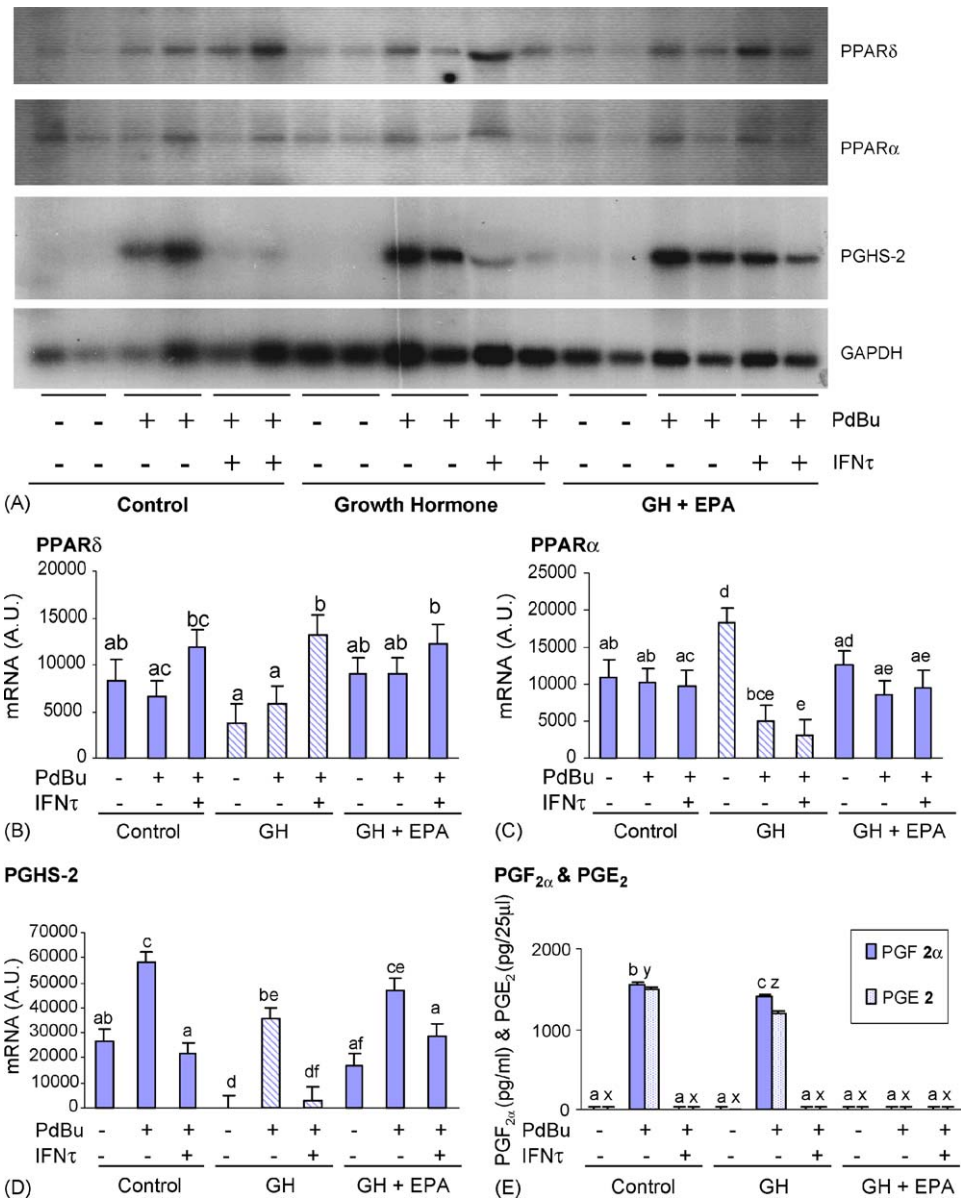


Fig. 2. Growth hormone and combined GH-EPA effects on PPARs and prostaglandin accumulation in BEND cells. (A) Total RNA was isolated from BEND cells and subjected to Northern blot analysis using specific bovine PPAR $\delta$ , PPAR $\alpha$ , PGHS-2 and GAPDH cDNA probes. GAPDH mRNA was used as a loading control. (B–D) Least squares mean  $\pm$  S.E. of indicated mRNA levels after adjustment for loading for two experiments each with duplicate samples. Treatments with different letters were different ( $P < 0.05$ ). (E) Accumulation of PGF $_{2\alpha}$  (pg/ml) and PGE $_2$  (pg/0.1 ml) in BEND cell supernatants was measured using RIA 3 h after PdBu treatment. Prostaglandin values are LS means  $\pm$  S.E. of a representative experiment performed in triplicate. Significant ( $P < 0.05$ ) differences among treatments are indicated by different letters within prostaglandin type.

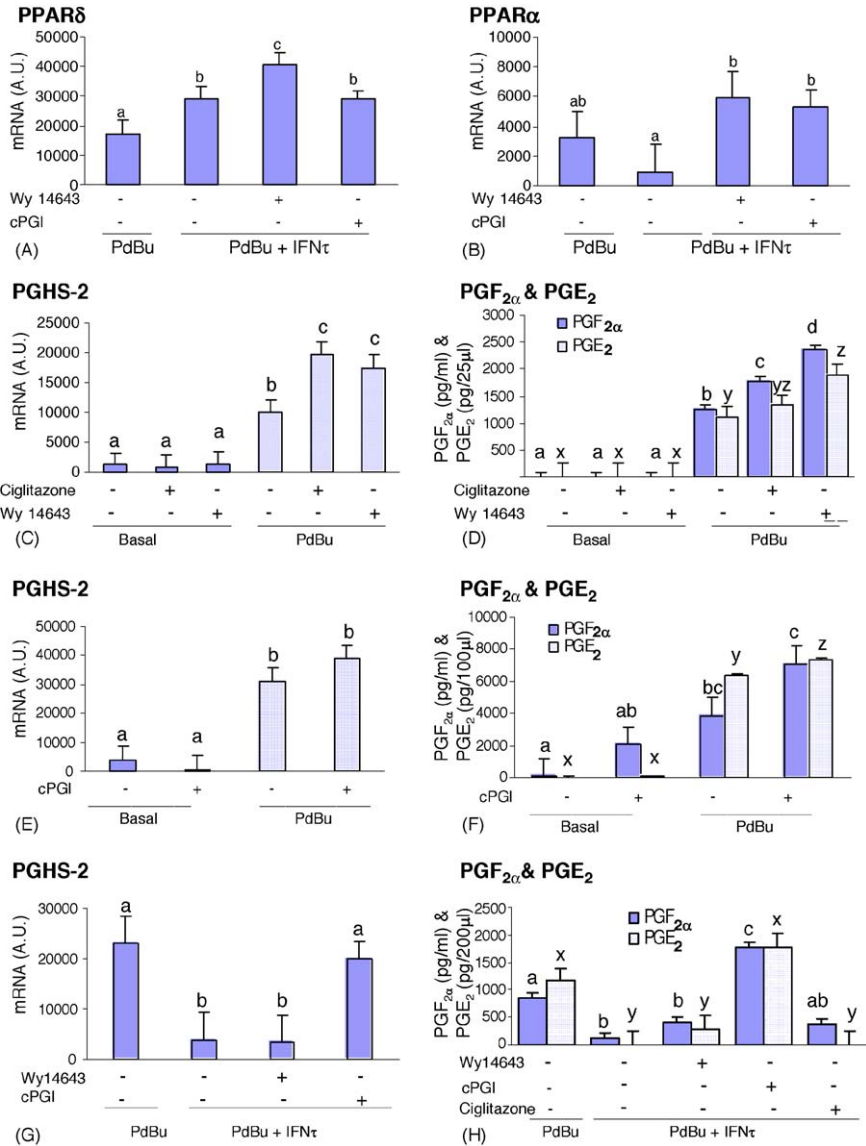


Fig. 3. Effect of PPAR agonists on BEND cell RNA levels and prostaglandin accumulation. Treatment of 10  $\mu$ M Wy14643 increased PPAR $\delta$  mRNA levels but treatment with 1  $\mu$ M cPGI did not (A). Either treatment increased PPAR $\alpha$  mRNA levels in PdBu-IFN $\gamma$ -treated cells (B). Treatment with 10  $\mu$ M ciglitazone or Wy14643 increased PGHS-2 mRNA levels and PG accumulation in BEND cells treated for 3 h with PDBu (C–D). Carbaprostacyclin (1  $\mu$ M) did not affect PGHS-2 mRNA levels but did increase PG accumulation in PDBu-treated cells (E–F). PGHS-2 mRNA levels and prostaglandin synthesis in PdBu-IFN $\gamma$ -treated cells were not affected by ciglitazone or Wy14643, but were stimulated by cPGI (G–H). Data are least squares mean  $\pm$  S.E. of two experiments each performed in duplicate. Treatments with different letters were different ( $P < 0.05$ ). Note different letter series used to differentiate prostaglandin types.

Growth hormone addition did not consistently affect PPAR $\delta$  mRNA levels ( $P > 0.05$ ; Fig. 2A and B). Basal PPAR $\alpha$  mRNA levels were increased by GH ( $P < 0.05$ ; Fig. 2C), but orthogonal contrasts indicated that there was a tendency for GH to decrease PPAR $\alpha$  mRNA levels in PdBu- and PdBu-IFN $\tau$ -treated cells in comparison to controls ( $P < 0.10$ ). Pretreatment with EPA prevented the GH effect (Fig. 2C). There was a small but significant decrease in PGHS-2 mRNA levels in response to GH in PdBu- and PdBu-IFN $\tau$ -treated cells ( $P < 0.05$ ; Fig. 2D). Combined GH and EPA treatment reversed the IFN $\tau$ -associated decrease in PGHS-2 mRNA levels ( $P < 0.05$ ). The effects of GH and EPA on PGHS-2 were subtle in comparison to those of PdBu and IFN $\tau$ .

Combining PdBu stimulation with either EPA, or EPA + IFN $\tau$  suppressed prostaglandin levels ( $P < 0.01$ ) such that they were not different from those observed in unstimulated cells (Fig. 2E). Treatment with GH alone reduced prostaglandin levels ( $P < 0.01$ ), but to a lesser extent than EPA or IFN $\tau$  ( $P < 0.05$ ; Fig. 2E).

### 3.4. Effects of PPAR agonists

In unstimulated or PdBu-treated BEND cells, treatment with the PPAR $\gamma$  agonist ciglitazone, with the PPAR $\alpha$  agonist Wy14643, or with carbaprostacyclin did not consistently influence PPAR $\delta$  or PPAR $\alpha$  mRNA levels ( $P > 0.05$ ; data not shown). In BEND cells treated with both PdBu and IFN $\tau$ , PPAR $\delta$  mRNA levels were stimulated by the PPAR $\alpha$  agonist Wy14643 ( $P < 0.05$ ; Fig. 3A), but not by ciglitazone (data not shown) or carbaprostacyclin ( $P > 0.10$ ; Fig. 3A). Both Wy14643 and carbaprostacyclin increased PPAR $\alpha$  mRNA levels in PdBu-IFN $\tau$ -treated cells ( $P < 0.05$ ; Fig. 3B).

Both the PPAR $\gamma$  and PPAR $\alpha$  agonistic compounds increased steady state levels of PGHS-2 mRNA in PdBu-treated cells (Fig. 3C;  $P < 0.05$ ), although the effect was modest in comparison to the PdBu effect. Treatment of BEND cells with PPAR agonists also affected prostaglandin production. Wy14643 stimulated PGF $_{2\alpha}$  and PGE $_2$  accumulation in the media above that stimulated by PdBu alone ( $P < 0.05$ ; Fig. 3D).

Carbaprostacyclin did not affect mRNA accumulation in BEND cells treated with PDBu (Fig. 3E), but tended to stimulate PGF $_{2\alpha}$  ( $P < 0.10$ ) and did stimulate PGE $_2$  ( $P < 0.05$ ) accumulation in the media of PdBu-treated cells (Fig. 3F). The IFN $\tau$  suppression of PGHS-2 mRNA in PdBu-treated cells was reversed by carbaprostacyclin (Fig. 3G;  $P < 0.02$ ). Carbaprostacyclin stimulated PGF $_{2\alpha}$  ( $P < 0.05$ ) and tended to stimulate PGE $_2$  ( $P = 0.10$ ) accumulation in PdBu-IFN $\tau$ -treated cells above that observed in PdBu-treated cells (Fig. 3H). Neither Wy14643 nor ciglitazone affected the prostaglandin responses to PdBu-IFN $\tau$  treatment.

## 4. Discussion

In this study, we show that PPAR $\delta$  is a likely mediator of some of the effects of long chain omega-3 PUFA action in bovine endometrium. PPAR $\delta$  appears to be constitutively expressed in BEND cells, consistent with our recent data indicating expression in the luminal and glandular epithelia of bovine endometrium (MacLaren et al., in preparation). The relative level of PPAR $\delta$  mRNA expression in bovine endometrial cells is under the influ-

ence of several factors, including the protein kinase C pathway (PdBu) and embryonic IFN $\tau$ . Stimulation of the protein kinase C pathway *in vivo* occurs in response to ligand binding to the oxytocin receptor to initiate pulsatile secretion of PGF $_{2\alpha}$  and consequent luteolysis. IFN $\tau$  suppresses this activity and prevents luteolytic PGF $_{2\alpha}$  pulses as part of the pregnancy recognition mechanism. Our observation that both PdBu and IFN $\tau$  stimulate PPAR $\delta$  mRNA accumulation while having opposite effects on prostaglandin synthesis suggest that the differentiation state of the cell is more important to series 2 prostaglandin output than the relative amount of PPAR $\delta$  mRNA.

The regulation of PPAR $\delta$  RNA expression is not well characterized. Inclusion of PdBu in the media also stimulates PPAR $\delta$  mRNA expression in normal keratinocyte cultures [22]. Ding and co-workers [23,24] showed that PPAR $\delta$  mRNA is regulated in uterine epithelium in early rodent pregnancy, and is induced in uterine subluminal stroma by the presence of the blastocyst. Levels of PPAR $\delta$  mRNA are elevated in cancerous colorectal cells, and it has been suggested that APC tumor suppressor activity and the nonsteroidal anti-inflammatory drugs reduce PPAR $\delta$  mRNA levels [25]. Fasting and refeeding significantly affects PPAR $\delta$  mRNA levels in kidney and liver, but not in other tissues [26]. Together, these studies indicate that PPAR $\delta$  gene expression is locally regulated, and that more research is warranted to clarify what role this regulation plays in mediating the actions of the receptor.

The BEND cell line responds to stimulation of protein kinase C (e.g., PdBu) or supplementation with EPA, GH and IFN $\tau$  in a manner consistent with *in vivo* observations, making it a useful model of bovine uterine prostaglandin synthesis. Regardless of treatment, both PPAR $\alpha$  and PPAR $\delta$ , but not PPAR $\gamma$ , mRNAs are expressed in these cells, as expected from expression patterns observed in whole endometrium (MacLaren et al., in preparation). Media accumulation of PGF $_{2\alpha}$  and PGE $_2$  occurs in parallel in this system regardless of treatments, suggesting that these two prostaglandins are co-regulated in BEND cells under the conditions tested, and that PPAR gene expression does not affect the ratio of these prostaglandins. Similarly, Arntzen et al. [8] found that production of both of these prostaglandins by human decidual cells were reduced by supplementation with omega-3 PUFA, and elevated by omega-6 PUFA.

As established previously [7], the suppression of prostaglandin synthesis by EPA does not depend upon a decrease in PGHS-2 mRNA levels. In contrast, EPA partially reverses the IFN $\tau$  suppression of PGHS-2 mRNA expression, which is consistent with the omega-3 PUFA stimulating transcription. Similarly, another omega-3 PUFA, docosahexanoic acid, has been shown to increase transcription of PGHS-2 mRNA in bovine endothelial cells [27]. Analysis of the bovine PGHS-2 promoter has been limited to the 1500 nucleotide region close to the start site, so it is not known whether it has a PPRE or DRE [28]. The human promoter has a functional PPRE located approximately 3700 bases upstream of the start site [13,14]. In addition to acting as a ligand for PPARs, long chain omega-3 PUFA such as eicosapentanoic acid and docosahexanoic acid can interact with other transcription factors, including hepatic nuclear factor 4 $\alpha$ , SREBP-1c, LXR, RXR $\alpha$  and NF $\kappa$ B, and can affect membrane phospholipid composition, including lipid rafts that are known to affect cell signaling from the plasma membrane [9]. It also is proposed that EPA may directly bind the PGHS-2 enzyme, inhibiting its function [10].

In human adipocytes, EPA, but not other PUFA, increases PPAR $\gamma$  mRNA levels [29]. It is also reported that activation of PPAR $\delta$  results in increased levels of PPAR $\gamma$  mRNA [30].

However, expression of PPAR $\gamma$  in BEND cells is not detectable by Northern analysis regardless of treatment. Despite this, some functional PPAR $\gamma$  must be present, since we observed that treatment of PdBu-treated BEND cells with the PPAR $\gamma$  specific agonist ciglitazone stimulated PGHS-2 mRNA levels and accumulation of both PGF $_{2\alpha}$  and PGE $_2$ . Induction of PGHS-2 mRNA by ciglitazone activation of PPAR $\gamma$  also occurs in cultured airway smooth muscle cells [14]. In these cells, this induction is overridden by dexamethasone, whereas in BEND cells, the ciglitazone induction of PGF $_{2\alpha}$  and PGE $_2$  is overridden by IFN $\tau$ . This pattern suggests that the PPRE influence on the promoter of PGHS-2 is weaker than that of other transcription factors involved in the response.

The PPAR $\alpha/\delta$  agonist carbaprostacyclin (cPGI) stimulates PGHS-2 mRNA and the accumulation of PGF $_{2\alpha}$  and PGE $_2$ , and this effect is observed even in the presence of IFN $\tau$ , which generally suppresses both PGHS-2 mRNA and prostaglandin accumulation. Carbaprostacyclin is considered an agonist of both PPAR $\delta$  and PPAR $\alpha$  [31]. Although the PPAR $\alpha$  agonist Wy14643 also stimulates PGHS-2 and prostaglandin accumulation in the absence of IFN $\tau$ , it has little effect in its presence, suggesting that PPAR $\alpha$  is not mediating the response to cPGI and that PPAR $\delta$  may be acting to counter the IFN $\tau$  effect on PGHS-2 expression. Similarly, transfection experiments in the human uterine carcinoma AN3CA cell line indicate that cPGI is not able to stimulate PPAR $\alpha$  [12]. It remains to be determined whether this limitation is specific to these cell lines or a characteristic of uterine tissues. It is interesting that *in vivo* there is an emerging importance of endogenous PGI $_2$  as a potent stimulator of PPAR $\delta$ . For example, in cultured medullary interstitial cells of the rabbit, PGI $_2$  acts through PPAR $\delta$  and its response element (DRE) to mediate the cell survival response seen in response to PGHS-2 induction by hypertonic stress [32]. In the mouse, PGI $_2$  is the most abundant prostaglandin at implantation sites. Both PGHS-2 and PGI synthase (PGIS) gene expression are induced at the time of implantation, yet the classic receptor for PGI $_2$ , IP, is not detected and the PGI $_2$  effects are thought to be mediated by PPAR $\delta$  [12]. PPAR $\alpha$  is not present in the rodent uterus at implantation, but expression of PPAR $\delta$  is induced at implantation in a pattern similar to PGHS-2 and PGIS [12,23,24]. Little is known about PGI $_2$  synthesis and activity in the ruminant uterus. Early studies indicated that there are few binding sites for PGI $_2$  in bovine endometrium, although PGIS is expressed in ovine endometrium and administration of PGI $_2$  increases endometrial blood flow [33–35].

PPAR $\alpha$  is expressed in the ovary and uterine epithelium of the rat [2], and we know from previous studies that growth hormone has an inhibitory effect on PGHS-2 mRNA and prostaglandin accumulation in BEND cells [16]. The current studies indicate that in unstimulated BEND cells, the expression of PPAR $\alpha$  mRNA is increased by exposure to growth hormone, whereas in the presence of the PdBu, growth hormone suppresses transcript levels. Pretreatment of BEND cells with EPA reverses the growth hormone depression of PPAR $\alpha$  and PGHS-2 mRNA levels. As is the case for the other PPARs, relatively little is known about the regulation of PPAR $\alpha$  mRNA. Cultured hepatocytes decrease mRNA levels of PPAR $\alpha$  in response to growth hormone, but this occurs over several days, not hours of treatment as we used here [36,37]. In rats, hepatic PPAR $\alpha$  mRNA expression decreases from 1–3 h post-growth hormone treatment, but recovers by 6 h post-treatment [38]. Interestingly, this effect is observed only in liver, and not in heart or soleus muscle, as is a sex difference in expression [38]. Liver expression of this mRNA in the rat is stimulated by fasting and by high circulating levels of glucocorticoids, and decreased by insulin [1,26,38].

Up-regulation of PPAR $\alpha$  mRNA is observed in response to its own ligands in other in vitro systems [1]. Given this apparent sensitivity of PPAR $\alpha$  transcript levels to a variety of hormones and the metabolic state of the cell, it is perhaps not surprising that growth hormone has different effects on cell PPAR $\alpha$  expression depending upon the particular intercellular pathways activated. Since the target genes of PPAR $\alpha$  are involved in cellular uptake, activation and  $\beta$ -oxidation of fatty acids, the growth hormone may augment this process in the BEND cells at rest by making more PPAR $\alpha$  available, and inhibit it in those that have an active PKc pathway.

In summary, we have demonstrated that PPAR $\alpha$  and PPAR $\delta$  are expressed in cultured bovine endometrial cells and are subject to regulation by factors known to regulate series 2 prostaglandin synthesis in these cells. The PPAR $\delta/\alpha$  agonist cPGI has a dramatic stimulatory effect on PGHS-2 mRNA levels and the synthesis of PGF $_{2\alpha}$  and PGE $_2$ , which appear to be mediated at least in part through PPAR $\delta$ . We hypothesize that PPAR $\delta$  is involved in the pregnancy recognition process of cattle and that it mediates at least some of the beneficial effects of long chain omega-3 PUFA supplementation.

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