RESEARCH ARTICLE

Supplementation of dairy cows with bovine somatotropin or omega-3 rich fish oil affects the endometrial expression of peroxisome proliferator-activated receptors

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Özet

MacLaren LA, Bilby TR, Michel F, Guzeloglu A, Staples CR, Thatcher WW. İnek somatotropini uygulaması veya omega-3 zengini balık yağı (FO) ile beslenen ineklerde peroxisome proliferator-activated reseptörlerinin (PPAR) ekspresyonunu etkiler. **Eurasian J Vet Sci, 2011, 27, 4, 207-218**

Amaç: Çalışmanın amaçları, holştayn ırkı ineklerin rasyonuna 90 gün boyunca zenginleştirilmiş balık yağı formunda uzun zincirli omega-3 çoklu doymamış balık yağı katılmasının veya ovulasyon günü ve 11 gün sonrasında iki kere inek somatotropin enjeksiyonunun endometriumunda PPAR ekspresyonu ve aktivasyonunu araştırmaktır.

Gereç ve Yöntem: Laktasyonda olmayan inekler siklik, siklik-bST, gebe veya gebe-bST olmak üzere 4 gruba ayrıldı. Laktasyondaki inekler ise siklik, siklik-bST, gebe, gebe-bST, siklik-FO veya siklik-FO-bST olmak üzere 6 gruba ayrıldı.

Bulgular: Northern ve Western blot analizleri PPARα ve PPARδ'nın ovulasyon sonrası 17. günde endometriumda ekspre edildiğini belirlendi ancak PPARγ tespit edilmedi. bST uygulaması sadece gebelerde PPARδ mRNA ekspresyonunu arttırdı, bu etki embriyo tarafından düzenlenmiş olabilir. Laktasyondaki gebe ineklerin endometriumundaki bST'ya bağlı PPARα mRNA miktarındaki artış bST etkisinin gebelik durumuna bağlı olduğunu vurgulamaktadır. Rasyona balık yağı eklenmesi PPARδ mRNA miktarını azaltırken, PPARα mRNA miktarı üzerine bir etkisi olmadı. PPARδ proteini luminal epitelde, glandular epitelde, subepitel stromada ve az miktarda da adluminal stromada belirlendi. Anti-PPARδ reaksiyonu bST uygulaması ve balık yağı beslemesine bağlı olarak gebe ineklerde azaldı.

Öneri: bST uygulaması ve balık yağı beslemesi endometrial PPARα ve PPARδ ekspresyonun laktasyondaki sütçü ineklerde etkilemektedir.

Abstract

MacLaren LA, Bilby TR, Michel F, Guzeloglu A, Staples CR, Thatcher WW. Supplementation of dairy cows with bovine somatotropin or omega-3 rich fish oil affects the endometrial expression of peroxisome proliferator-activated receptors (PPARs). **Eurasian J Vet Sci, 2011, 27, 4, 207-218**

Aim: The study objectives were to determine whether dietary supplementation with long chain omega-3 polyunsaturated fatty acids in the form of enriched fish oil (FO) for 90 days or treatment with bovine somatotropin (bST) at the time of ovulation and 11 days post-ovulation influenced PPAR expression and activation in bovine endometrium in Holstein cows.

Materials and Methods: Non-lactating cows were assigned to one of four treatments: cyclic, cyclic-bST, pregnant or pregnant-bST. Lactating cows were assigned to one of six treatments: cyclic, cyclic-bST, pregnant, pregnant-bST, cyclic-FO or cyclic-FO-bST.

Results: Northern and Western blot analyses indicated that PPARα and PPARδ, but not PPARγ, are expressed in endometrium from all cows at day 17 post-ovulation. Treatment with bST is associated with increased PPARδ mRNA abundance in pregnant but not cyclic cows, suggesting that the effect may be mediated by the embryo. Increased abundances of PPARα mRNA are observed in response to bST during pregnancy in lactating cows but not in non-lactating cows, highlighting the importance of lactation status in determining bST response. Fish oil supplementation is associated with reduced PPARδ mRNA abundance, but did not affect steady-state PPARα mRNA abundance. PPARδ protein is expressed in the luminal epithelium, glandular epithelium, subepithelial stroma and to a lesser extent in the adluminal stroma. Anti-PPARδ reactivity is reduced in response to bST and fish oil treatments in pregnant cows.

Conclusion: bST and fish oil treatments affect endometrial PPARα and PPARδ expression in lactating dairy cows.

► Introduction

Pregnancy rates in dairy cattle are increased when lactating cows are fed diets high in the omega-3 long chain polyunsaturated fatty acids (n-3 PUFA) or if they are treated with recombinant bovine somatotropin (bST) at the time of insemination (Burke et al 1997, Moreira et al 2002, Santos et al 2004, Thatcher et al 2006, Silvestre et al 2011). It is proposed that n-3 PUFAs reduce embryo mortality in part by preventing the pulsatile release of endometrial prostaglandin $F_{2\alpha}$ that preceeds luteolysis at approximately 17 days postpartum (Thatcher et al 2006). It is well established that n-3 PUFA are incorporated into reproductive tissues and are often associated with reduced capacity for series 2 prostaglandin synthesis (Mattos et al 2002, Wamsley et al 2005, Bilby et al 2006a, Perez et al 2006). Whether the effect on uterine prostaglandin synthesis is inhibitory or neutral appears to depend upon the balance of PUFA fed, as well as other management factors (Burke et al 1997, Wamsley et al 2005, Perez et al 2006). Several routes of action are proposed to explain the inhibitory effect of n-3 PUFA on series 2 prostaglandin synthesis, including competition with arachidonic acid for the enzyme prostaglandin synthase-2 (PGHS-2) and direct inhibition of the PGHS-2 enzyme by EPA (Mattos et al 2002). However, n-3 PUFA are known in other tissues to influence gene expression more widely (Desvergne and Wahli 1999, Feige et al 2006), and it is unlikely that it is only endometrial prostaglandin synthesis that is affected by these compounds.

Growth hormone (somatotropin) also has broad metabolic effects, including several mediated by insulin-like growth factor (IGF-1). Higher circulating concentrations of IGF-1 are found in cows treated with bST, and early embryo development is increased (Bilby et al 2004, Bilby et al 2006b). Expression of genes known to influence endometrial accommodation of pregnancy also is altered by bST treatment (Guzeloglu et al 2004, Bilby et al 2006c) but the routes of action of the hormone are unknown. The beneficial effects of bST treatment appear to be restricted to cows in lactation, emphasizing the importance of considering metabolic status in determining biological response to exogenous treatments, and providing an interesting model for assessing biological actions (Bilby et al 2004, Bilby et al 2006b).

A potential target of bST and n-3 PUFA is the peroxisome proliferator activated receptor (PPAR) family, which includes the three nuclear receptors PPARα, PPARδ and PPARγ. PPARα is highly expressed in tissues utilizing fatty acids as energy sources, and is known to regulate transcription of a number of genes associated with lipid metabolism (Desvergne and Wahli 1999, Escher et al 2001, Feige et al 2006). PPARδ (also known as PPARβ) is widely expressed and appears to be associated with differentiation and development in a variety of cell types (Desvergne and

Wahli 1999, Escher et al 2001, Burdick et al 2006, Feige et al 2006). The two isoforms of PPARγ are normally associated with adipose tissue and metabolism, but are expressed in ovary and placenta as well (Escher et al 2001, Cui et al 2002, Fournier et al 2007). Upon activation, PPARs heterodimerize with the RXR receptor and bind to PPAR response elements. PPAR activation may be ligand dependent or independent, and there is cross-talk with the other nuclear receptors and their response elements, as well as several transcription factors (Nunez et al 1998, Desvergne and Wahli 1999, D'Eon et al 2005, Feige et al 2006). PPAR response elements have been described on several genes associated with lipid metabolism, as well as the prostaglandin synthetic enzyme prostaglandin synthase-2 (Meade et al 1999). There are differences in ligand specificity of the PPAR isoforms, but all are promiscuous and variably activated by a number of long chain fatty acids, eicosanoid and fibrate ligands. The polyunsaturated long chain fatty acids are natural ligands for all three PPARs, including the n-3 PUFA derived from fish oils such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) (Krey et al 1997, Sethi et al 2002). Estradiol, fasting and growth hormone have all been shown to affect PPAR expression in different cell systems (Carlsson et al 2001, Escher et al 2001, D'Eon et al 2005, Faddy et al 2006).

Although both n-3 PUFA and growth hormone are known to have uterine effects, their relationship to PPAR expression in the ruminant has not been studied. The PPARs are expressed in rodent and ovine uteri (Escher et al 2001, Cammas et al 2006), and have been linked to reproductive function (Lim et al 1999, Cui et al 2002, Fournier et al 2007).

We hypothesize that PPARs mediate in part the bovine endometrial response to supplemental bST and n-3 PUFA-enriched feeds. The objectives of this study were to determine whether n-3 PUFA supplementation or bST treatment beginning at insemination influenced PPAR expression and activation in bovine endometrium, and to characterize PPAR expression in relation to pregnancy and lactation status.

► Materials and Methods

• *Animals and Experimental Design*

Two experiments were carried out with Holstein cows at the University of Florida Dairy Research Unit under the approval of the University of Florida Animal Care Committee. For experiment 1, mature, non-lactating Holstein cows were assigned in a 2 x 2 factorial design to one of four treatments: Cyclic (C), Pregnant (P), Cyclic plus bovine somatotropin (C-bST, 500 mg bST by intramuscular injection (i.m.) at day 0 (estrus or day of timed insemination) and again at day 11, or Pregnant plus bST (P-bST) at day 0 and again at day 11. Detailed descriptions of treatments are provided elsewhere (Bilby et al 2006b). Briefly, all cows were injected on Day -10 with GnRH (86 μg i.m. Fertagyl®,

Intervet Inc., Milsboro DE) followed 7 days later (Day -3) by an injection of $PGF_{2\alpha}$ (25 mg, i.m. Dinoprost Tromethamine, Lutalyse®, Pharmacia, Kalamazoo, MI). At 48 h after injection of $PGF2_{\alpha}$, GnRH (Day -1) was administered, and 55 cows were inseminated 16 h later (day 0). The cyclic groups (n=23) were not inseminated. Treatment group final numbers were C: n=7, P: n=7, C-bST: n=7, P-bST: n=9.

For experiment 2, mature, lactating Holstein cows were assigned in a incomplete randomized design to one of six treatments following calving: Cyclic (C), Pregnant (P), Cyclic plus bST (C-bST), Pregnant plus bST (P-bST), Cyclic plus Fish Oil (C-FO), or Cyclic plus Fish Oil and bST (C-FO-bST). Cows treated with bST received 500 mg i.m. on day 0 and again at day 11; details provided in Bilby et al (2004). The ruminally protected fish oil diet included 1.9% calcium salt of fish oil-enriched lipid supplement (EnergG-II Reproduction Formula,Virtus Nutrition, Fairlawn, OH) fed so that cows consumed approximately 15 g/day of EPA plus DHA. All diets were fed in a total mixed ration, were isocaloric and isonitrogenous, and based on NRC requirements for healthy dairy cows. The diets were fed from day 10 after parturition until the end of the experiment (94±12 days postpartum). Cows were pre-synchronized to ensure animals were between days 5 and 12 of the estrous cycle at the start of the timed AI protocol, which was carried out as described above. Cows assigned to cyclic treatments were not inseminated, whereas those assigned to pregnancy groups were inseminated on day 0. Treatment group final numbers were C: n=5, C-bST: n=6, P: n= 4, P-bST: n=5, C-FO: n=4, C-FO-bST: n=4.

For both experiments, all cows were slaughtered on Day 17 post-estrus. Reproductive tracts were collected within 10 minutes of exsanguination and pregnancy was confirmed by the presence of the conceptus. Inseminated cows that did not become pregnant were eliminated from the experiments. The uterus was flushed with PBS prior to dissection of the endometrial tissue from the anti-mesometrial border of the ipsilateral horn. The tissue was frozen in liquid nitrogen (for protein and RNA extraction), or fixed in 4% paraformaldehyde in PBS for immunohistochemistry. Single samples of kidney and adipose tissues also were collected from randomly selected cows at the abattoir to use as positive control tissues.

• *Extraction of RNA and Northern Blots*

The relative abundance of PPAR mRNAs were assessed in bovine endometrial samples by Northern blotting. Total RNA was isolated from endometrial tissues using Trizol® according to the manufacturer's recommendations (Invitrogen Corporation, Carlsbad, CA), then quantitated by spectrophotometry. Intron-spanning primers were designed to amplify cDNA from mRNA transcribed by the bovine PPARα, PPARδ and PPARγ genes (MacLaren et al 2006). For PPARγ,

the primers recognized a sequence common to both PPARγ1 and PPARγ2 gene products. Total RNA (1 µg) from kidney (for PPARδ and PPARα) and adipose (for PPARγ) 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 µL of the cDNA reaction product in a 50 µL reaction mix containing Taq polymerase (Boehringer-Mannheim) in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf Scientific Inc., Westborg, NY). The PCR products were subcloned into TOPO® vector (Invitrogen).

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 0C, and then hybridized with random primed ^{32}P-labelled cDNA probes for either PPARα, PPARδ or PPARγ overnight at 42 °C. The next day, the blots were washed in 2X SSC/0.1 % SDS and twice in 0.1X SSC/0.1 % SDS for 20 min each at 42 °C. The blots were then exposed to x-ray film. Blots were stripped with 1% SDS, and then reprobed with a cDNA specific for bovine glyceraldehyde-3-phosphate dehydrogenase (GapDH) mRNA to use as a housekeeping control for RNA loading. Samples from all cows within an experiment were run on two gels on the same day, blotted and probed in parallel using aliquots of the same solutions for all procedures. Densitometry (Alpha Imager 2000, Alpha Innotech Corporation, San Leandro, CA) was used to compare pixel intensity of PPAR and GapDH transcripts within each experiment.

• *Western Blots*

Endometrial tissue (300 mg) was sonicated 3 times for 5 sec each in 2 mL of whole cell extract buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM NaF, 1 mM Na-3VO4, 1 mM Na4P2O7, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF; 10% v/v glycerol, 1% v/v NP-40, and 10 µg/mL each of aprotinin, leupeptin, and pepstatin). Lysates were centrifuged (14000 x g for 10 min), and protein concentrations determined in supernatants. Protein samples (100 µg) from all cows within an experiment were electrophoresed in 10% denaturing SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 2 h in 5% (w/v) nonfat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), washed for 15 min in TBST, and probed with goat antibody to the amino terminus of PPARα (1:333, catalogue #sc-1985, Santa Cruz Biotechnologies Inc., Santa Cruz, CA) or rabbit antibody to the amino terminus of PPARδ(1:333, catalogue # sc-7197, Santa Cruz Biotechnologies)diluted in 5% nonfat dried milk in TBST for 2 h. Secondary antibodies were HRP-conjugated anti-goat IgG (Santa Cruz Biotechnologies)

or HRP-conjugated anti-rabbit IgG (Amersham Corp., Arlington Heights, IL) diluted in 5% nonfat dried milk in TBST. Proteins were detected using a chemiluminescent substrate (Renaissance Western Blot Chemiluminescent Reagent Plus, NEN Life Science Products, Boston, MA) and analyzed by densitometry (Alpha lmager 2000).

• *Immunohistochemistry*

For immunohistochemical localization of PPARδ, paraffin sections (5 μm) from the anti-mesometrial border of the uterus from all lactating cows (experiment 2) were prepared. Following deparaffinization, antigen retrieval was performed by heating sections in a microwave oven at high power for 5 min in 0.01 M sodium citrate buffer (pH 6.0). Sections were allowed to cool for 20 min and then washed in phosphate buffered saline (0.01 M PBS, pH 7.5). Nonspecific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in methanol for 10 min at RT. After a 10-min wash in PBS, non-specific binding was blocked with 5% normal goat serum in PBS in a humidified chamber at RT for 1 h. The tissue sections were then probed for 2 h at RT with affinity purified rabbit antibody to the amino terminus of human PPARδ (Catalogue # sc-7197, Santa Cruz Biologicals). Adjacent sections were incubated with rabbit IgG at the same concentration as the primary antibody to serve as a negative control. Following incubation with primary antibodies, immunoreactive protein was detected with an anti-rabbit ABC detection kit (Vector Laboratories, Burlingame, CA). The sections were counterstained with hematoxylin and dehydrated before mounting with Permount® (Fisher).

Image analysis was performed to estimate the relative abundance of PPARδ staining in different cell types. Treatment-blind assessment of immunostaining was carried out on the following endometrial compartments of 7-10 randomly selected fields of intercaruncular regions in three pieces of endometrium from each cow: luminal epithelium (LE), superficial glandular epithelium (GE), deep glandular epithelium (DGE), subepthelial stroma (S), adluminal stroma (DS). Caruncular endometrium was not evident in all cows, but luminal epithelium (CLE), subepithelial (CS) and adluminal stroma (CDS) were scored where possible. The intensity of nuclear staining was scored on a 4-point scale where 0=no staining (no brown), 1=light (light brown), 2=moderate (brown) and 3=heavy (dark brown), and the staining intensities were expressed as percentage of positively stained cells for each point in the scale (Guzeloglu et al 2004).

• *Electrophoretic Mobility Shift Assay (EMSA)*

Preparation of nuclear extracts for use in the EMSA was adapted from Liu et al (Liu et al 1995). Briefly, 0.5 g of endometrial or kidney (positive control) tissues were chopped and homogenized in buffer containing 10 mM HEPES, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM

DTT, and 0.1% NP40. Following centrifugation, the nuclear pellet was lysed with 60 μL of lysis solution containing 20 mM HEPES, 1.5 mM MgCl$_2$, 0.42 mM NaCl, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF and 0.2 mM EDTA. Samples were re-centrifuged, and the soluble fraction mixed with 100 μL of buffer containing 20 mM HEPES, 50mM KCl, 0,5 mM DTT, 0.5 mM PMSF and 0.2 mM EDTA before determination of protein concentration and storage at –80^{0}C.

Two oligonucleotide templates were used. The classic PPAR response element (PPRE) was contained in the first oligonucleotide, 5'-CAAAACTAGGTCAAAGGTCA-3' (Catalogue sc-2583, Santa Cruz Biotechnologies). The second oligonucleotide was 5'-GCGTGAG<u>C-GCTCACAGGTCA</u>ATTCG-3', which contains the PPARδ response element (DRE) identified, by He et al (1999). The DNA templates were end-labeled using ^{γ32}P-ATP prior to use in the EMSA.

Aliquots of nuclear extracts (10 μg) were mixed with 2 μL poly(dI-dC) and incubated in a 40-μl reaction volume containing 10 mM Tris-Cl (pH 7.4), 60 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 10% (w/v) glycerol and 0.1 μg/μL sonicated herring sperm DNA for 20 min at 37^{0}C. The DNA template (^{γ32}P-labeled PPRE or DRE) was then added and incubation was continued for 10 min at RT. For negative controls, excess cold template was added prior to the initial incubation. DNA-protein complexes were separated on a 6% non-denaturing polyacrylamide gel in standard TAE buffer and detected by autoradiography. Band intensities were determined by densitometry as indicated above.

• *Data Expression and Statistical Analysis*

For each experiment, effects of treatment on PPAR RNA, protein and protein-DNA complex responses were determined by analysis of variance using the general linear models procedure in SAS™ (SAS Institute Inc. Cary NC). For Northern blot analyses, blot was a factor and the pixel intensity of the GapDH transcript nested within blot was included as a covariate in the model. Blot by treatment effects were not significant. For the immunoblots, a control sample was scanned on each blot and its signal intensity used as a factor in the model to correct for variation among blots. A predetermined series of orthogonal contrasts for treatment examined effects of pregnancy status, bST treatment and bST-pregnancy status interaction for experiment 1, and effects of pregnancy status, bST, and bST-pregnancy status interaction, or fish oil supplementation, bST and bST-fish oil supplementation for experiment 2. The selected α error rate was p≤0.05.

Data generated from immunohistochemistry were analyzed by the mixed model procedure of SAS for each cell compartment. The model included treatment (C, P, C-bST, P-bST, C-FO, C-FO-bST), scoring class (0-no staining, 1-light, 2-moderate, 3-heavy) and treatment
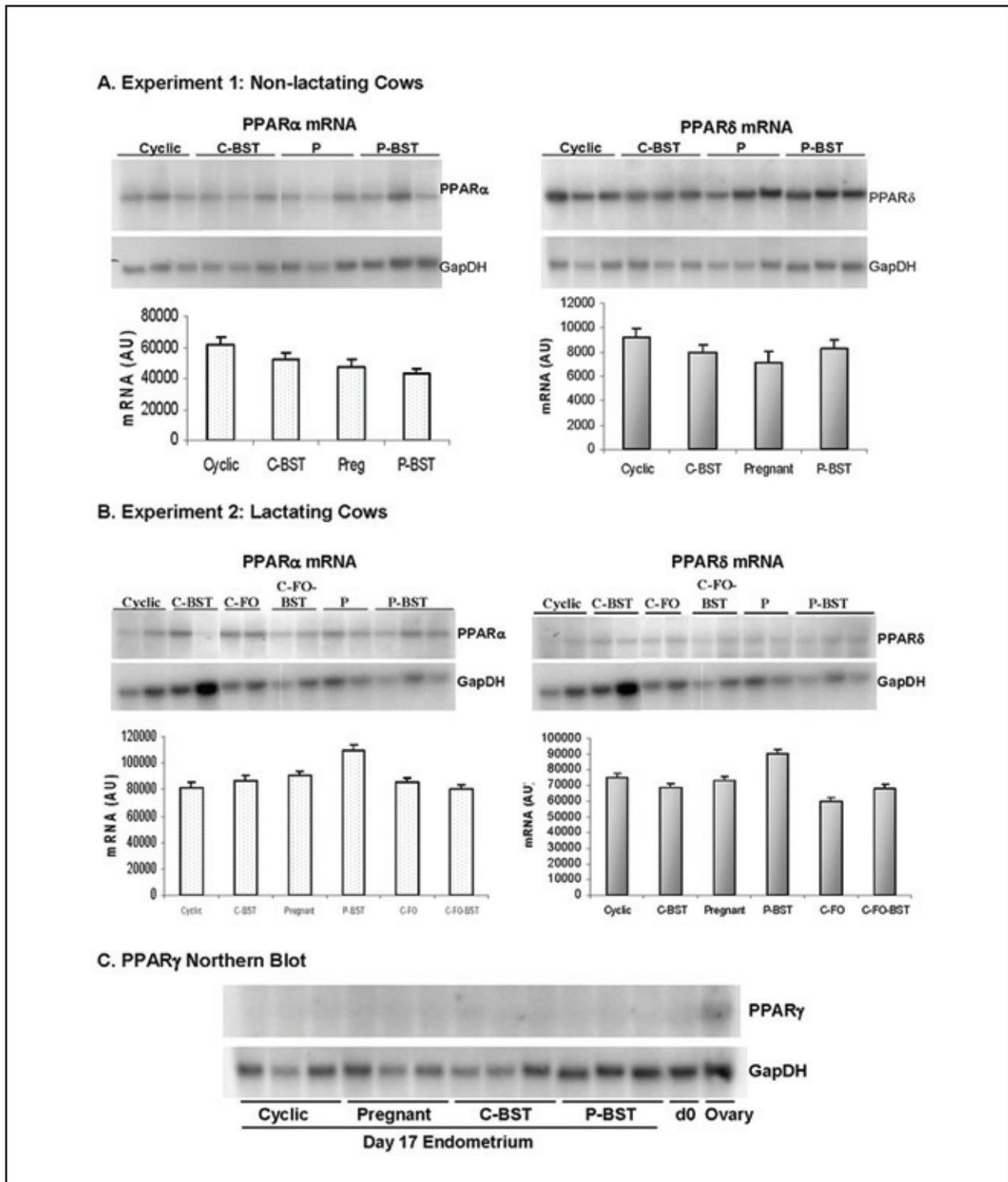
Figure 1. Results of Northern blots of total endometrial RNA using ³²P-labelled cDNA probes specific for PPARα, PPARδ , PPARγ or GapDH. A. Sample Northern blots and summary of least squares means ± standard errors for abundances of PPARα and PPARδ steady state mRNA by treatment, expressed as arbitrary units. RNA samples were from non-lactating cows that were either cyclic (C) or pregnant (P), with or without bST treatment at insemination (C-bST, P-bST). B. Sample Northern blots and summary of least squares means ± standard errors of relative steady-state mRNA abundances in endometrium from lactating cows by treatment. FO indicates supplementation with fish oil through early lactation. The blot shown was probed simultaneously with PPARα and PPARδ, then re-probed with GapDH.C. Sample Northern blot probed with PPARγ and GapDH.

by scoring class interactions. Cow nested within treatment was used as the error term for treatment effects. A series of orthogonal contrasts for treatment examined the effects of pregnancy status, bST treatment and bST-pregnancy status, or fish oil supplementa-

tion, bST and interaction of bST-fish oil supplementation on the proportions of scores classed in 0/1 vs 2/3, 0 vs 1, and 2 vs 3.

► **Results**

• *Northern Blots*

Northern blot analysis of endometrial extracts revealed expression of a single transcript for bovine PPARα of approximately 10 kilobases (kb) in length and a single transcript for bovine PPARδ of approximately 4 kb (Figures 1A-B). These transcript sizes correspond to the previously observed sizes of the bovine PPARα and PPARδ mRNAs (MacLaren et al 2006). Reactivity of PPARγ mRNA in endometrial samples was weak or undetectable by Northern blotting. However, samples of ovarian or adipose mRNA indicated that our probe recognized two transcripts, approximately 2.1 and 2.3 kb, in those tissues as expected from previous studies (Figures 1C) (Sundvold et al 1997).

The transcripts for PPARα and PPARδ were observed in endometrium from all cows, regardless of lactational status or experimental treatment, and relative expression responses were similar in terms of times required for band exposure and pixel intensities (Figures 1A-B). Lactation status impacted the influence of treatments on PPARα steady-state mRNA abundanc-

es. In non-lactating cows, PPARα mRNA abundances were lower in pregnant or bST-treated animals (pτ0.05), whereas in lactating cows, pregnancy and bST treatment at insemination were associated with increased PPARα mRNA abundances (pτ0.05, Figures 1A-B). In lactating cows, there was a pregnancy status by bST treatment interaction (pτ0.05) so that PPARα mRNA abundance was highest in pregnant animals treated with bST. Both dietary fish oil supplementation and bST failed to impact PPARα mRNA abundances in endometrium from cyclic, lactating cows (p>0.10, Figure 1B).

The influences of pregnancy and bST treatment on PPARδ steady state mRNA abundances were similar in non-lactating and lactating cows (Figure 1). Treatment with bST coincided with lower abundance of endometrial mRNA for PPARδ in cyclic cows, whereas bST stimulated PPARδ abundance in pregnant cows (for nonlactating cows, bST by pregnancy status interaction P=0.08; for lactating cows, interaction pτ0.01). Cyclic, lactating dairy cows that were fed the fish oil supplemented diet had lower abundance of endometrial PPARδ mRNA than unsupplemented cows
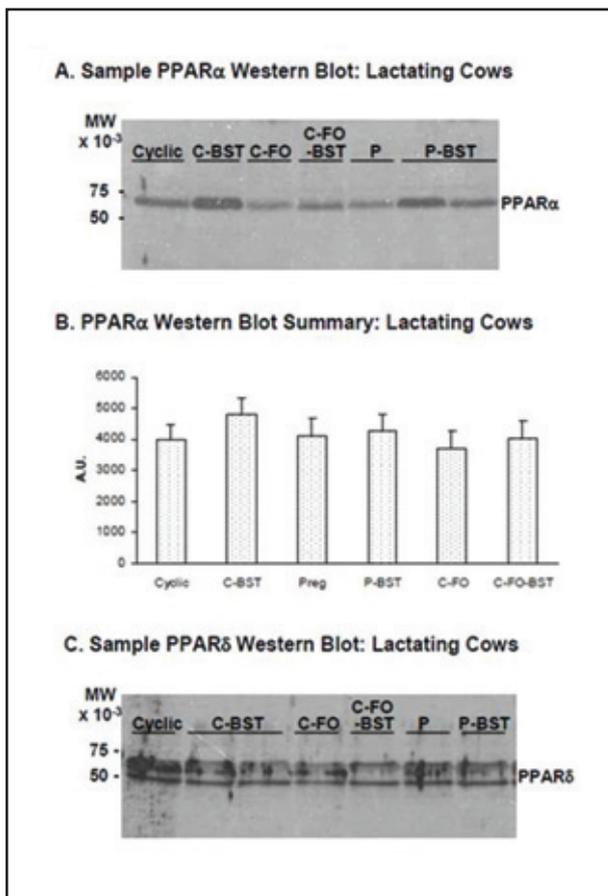
Figure 2. Western blot analysis of PPAR protein expression in day 17 endometrium from lactating cows. A. Representative Western blot of PPARα endometrial protein demonstrating a single immunoreactive band at 60 kDa. B. Least squares means ± standard errors of PPARα protein expression in endometrium from lactating cows, expressed as arbitrary units. C. Representative Western blot of PPARδ endometrial protein, indicating three reactive bands at 73, 68 and 55 kDa.
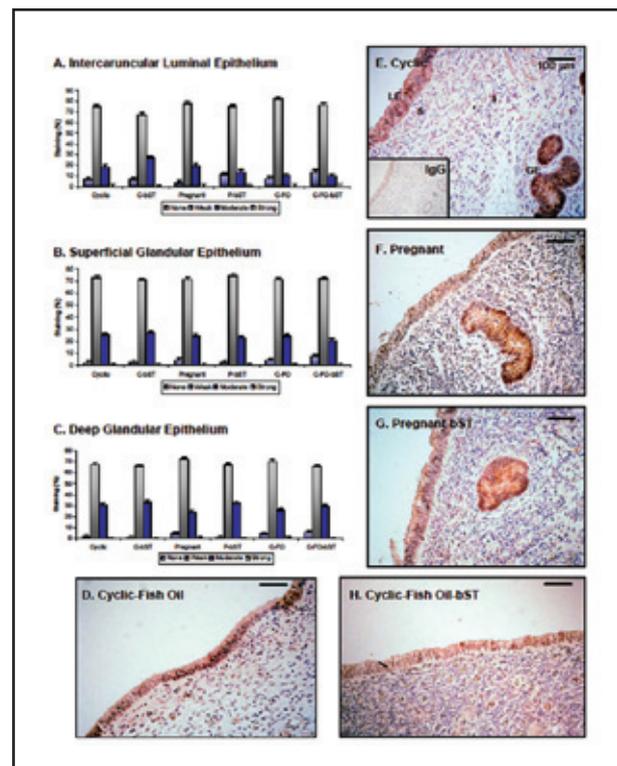
Figure 3. Localization and relative expression of PPARδ protein in day 17 endometrium from lactating cows. Panels A, B and C indicate the mean proportions of cells expressing no (0), light (1), moderate (2), or heavy (3) staining within each treatment for the indicated tissue compartment. Panels D through I show PPARδ antibody reactivity (brown nuclear staining) in representative endometrial tissue sections from lactating cows on indicated treatments. Inset in E indicates results when rabbit IgG is substituted for primary antibody. LE-luminal epithelium, S-subepithelial stroma, GE-glandular epithelium. Bar=100μm.

(pτ0.05). This effect did not occur in bST-treated cyclic cows fed the fish oil supplemented diet, reflecting a bST-diet interaction (pτ0.05).

• *Western Blots of PPARs and Localization of PPARδ*

Western blotting for PPARα in endometrial whole cell extracts detected a single band of approximately 55 kD (Figure 2A). This corresponds to the size of the full-length human protein observed in previous studies and suggests that carboxy-terminal truncated forms of PPARα are not present in bovine endometrium (Cernuda-Morollon et al 2002). Significant differences in PPARα protein expression were not detected among treatments in either non-lactating or lactating cows (Figure 2B), and all cows expressed the protein. Using an antibody to the N-terminus of PPARδ, three bands were consistently detected in endometrial protein extracts of approximately 55, 68 and 73 kDa, respectively, although treatment differences in band intensity were not detected (Figure 3C). Based on studies of human cell lines, the molecular mass of PPARδ protein is expected to be approximately 55 kDa (Cernuda-Morollon et al 2002).

Immunohistochemistry was performed to localize PPARδ but not PPARα since reactive antibodies could not be identified, despite extensive effort. PPARδ was expressed in the luminal epithelium of both caruncular and intercaruncular regions of the endometrium, as well as in the glandular epithelium and stroma (Figure 3). Expression was restricted to the nuclei, as expected for this receptor. Overall, approximately 70% of cells in any given field showed light (score 1) reactivity to PPARδ antibody, regardless of cell type (Figures 3A-C). There were subtle but significant differences among proportions of cells scoring in each class between treatments (Figures 3A-C). Table 1 summarizes the probabilities of differences between the indicated treatments scores within endometrial cell compartment. In the intercaruncular luminal epithelium (LE), there was a bST by pregnancy status interaction (pτ0.05) such that bST was associated with increased PPARδ expression in cyclic cows but not in pregnant cows. There was also a bST by fish oil interaction (pτ0.05) in that PPARδ moderate reactivity was reduced in fish-oil treated cows compared to control cyclic cows, and bST stimulated expression in control cyclic cows. The pattern of fish oil suppression of PPARδ expression also was evident in steady state abundance of PPARδ mRNA (Figure 1B). Similar patterns were observed in caruncular luminal epithelium (CLE), but presumably because of smaller numbers of observations (not all sections observed contained caruncular endometrium), fewer contrasts indicated significant changes (Table 1). In the superficial glandular epithelium (GE), few contrasts were significant although the proportion of cells scoring 0 or 1 vs 2 or 3 was different in fish oil supplemented cows (i.e., proportion 2 + 3 was lower, pτ0.05), and fish oil-bST treated cows tended to have a further reduction in

moderate scoring percent (FO x bST interaction for 0/1 vs 2/3, P=0.07). In the deep glands (DGE), contrasts indicated that staining was reduced in response to pregnancy (0/1 vs 2/3, pτ0.05) or fish oil (0/1 vs 2/3, pτ0.05). Unlike what was observed in the luminal epithelia, bST was associated with increased staining intensity in the deep glands (0/1 vs 2/3, Pτ0.05). The intercaruncular and caruncular stroma showed few differences in staining patterns associated with treatments (Table 1), perhaps because staining was so weak overall (Figure 3D-H).

• *Electrophoretic Mobility Shift Assays*

Nuclear extracts prepared from endometrial samples of 12 lactating cows, representing the six treatments of experiment 2, bound both the PPAR response element (PPRE) and the PPARδ response element (DRE, Figure 4). Two bands were observed in EMSAs utilizing the PPRE (Figure 4A). The higher molecular weight product was also evident in extracts prepared from kidney, which was used as a positive control, but the lower molecular weight complex was present only in the endometrial samples. Protein binding to the PPRE was observed in all samples, and there was a trend towards increased binding intensity in samples from cyclic bST-treated animals (Figure 4A, Upper band: C and C-FO vs C-bST and C-FO-bST, P=0.10).
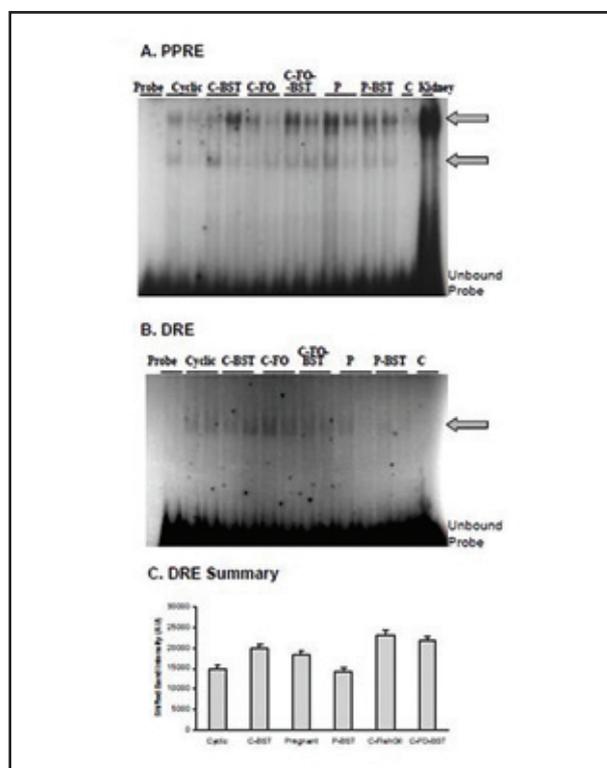
Figure 4. Binding of nuclear proteins in day 17 bovine endometrium to A, the PPAR response element (PPRE) and to B, the specific PPARδ response element (DRE). No extract was included in lanes labeled probe. C-competitor (cold probe) added as a negative control. Arrows indicate specific binding. C. Least squares means ± standard errors of protein-DRE complex band intensities by treatment.

Table 1. Probabilities for PPARδ antibody reactivity score contrasts among treatment groups for cyclic and pregnant lactating cows supplemented with bST or fish oil.

Treatment Contrast	Score Contrast	Pregnancy Status	bST	bST – Pregnancy Interaction	bST in Cyclic Cows	Fish Oil	Fish Oil-bST Interaction
		C+C-bST vs P+P-bST	C+P vs C-bST + P-bST	C+P-bST vs C-bST + P	C+C-FO vs C-bST +C-FO-bST	C+C-bST vs C-FO + C-FO-bST	C+ C-FO-bST vs C-bST + C-FO
Intercaruncular endometrium							
Luminal Epithelium	0 vs 1	.19	.01	.67	.01	.21	.53
	0/1 vs 2/3	<.01	.41	<.01	.03	<.01	.02
	2 vs 3	.09	.59	.04	.22	<.01	.13
Superficial Stroma	0 vs 1	<.01	.77	.42	.31	.20	.12
	0/1 vs 2/3	.33	.81	.38	.77	.62	.11
	2 vs 3	.74	.87	.49	.99	.65	.37
Deep Stroma	0 vs 1	.02	.96	.74	.91	.14	.68
	0/1 vs 2/3	.91	.03	.08	.07	.41	.02
	2 vs 3	.76	.21	.29	.28	.53	.17
Superficial Glandular Epithelium	0 vs 1	1.00	.72	.19	.32	.17	1.00
	0/1 vs 2/3	.17	.96	.32	.50	.03	.08
	2 vs 3	.47	.97	.56	.69	.22	.33
Deep Glandular Epithelium	0 vs 1	.54	.63	.87	.21	.48	.35
	0/1 vs 2/3	.01	<.01	.07	.04	<.01	.67
	2 vs 3	.20	.09	.34	.28	.12	.78
Caruncular endometrium							
Luminal Epithelium	0 vs 1	<.01	<.01	<.01	<.01	.01	<.01
	0/1 vs 2/3	<.01	.32	.26	.92	.01	.82
	2 vs 3	.14	.59	.61	.88	.18	.90
Superficial Stroma	0 vs 1	.04	.02	.71	.15	.27	.17
	0/1 vs 2/3	.17	.37	.81	.55	.63	.96
	2 vs 3	.45	.60	.94	.73	.66	.92
Deep Stroma	0 vs 1	.85	.09	.28	.40	.03	.05
	0/1 vs 2/3	.72	.57	.91	.53	.38	.87
	2 vs 3	.86	.68	.93	.78	.71	.97

[1]Within each tissue compartment, least squares means of scores for antibody reactivity (0-none, 1-weak, 2-moderate, 3-strong) were tested for the indicated treatment effects using orthogonal contrasts (score 0 vs 1, scores 0/1 vs 2/3, and score 2 vs 3). Significant contrasts (P≤0.05) are listed in bold type, whereas contrasts for which P<0.10 are listed in bold italics.

One protein-DNA band was observed when extracts were probed with DRE (Figure 4B). Both fish oil and bST appeared to increase protein-DRE binding in cyclic cows, but the effect was not additive resulting in a fish oil-bST interaction (pτ0.05, Figure 4C). There was also a significant pregnancy by bST interaction (pτ0.05) indicating that bST enhanced DRE binding in endometrium of cyclic cows but bST reduced DRE binding in endometrium of pregnant cows.

► **Discussion**

The results of these experiments suggest that PPARα and PPARδ are targets for omega-3 PUFA and bST action in bovine endometrium. The mRNAs and proteins from PPARα and PPARδ were readily detected in endometrium from all animals, whereas PPARγ mRNA was not evident in endometrium regardless of treatment or pregnancy status.

Treatment with bST at insemination and 11 days later was associated with increased endometrial PPARδ mRNA abundance at day 17 of pregnancy, regardless of lactation status. The bST effect was not seen in cyclic cows. One consequence of bST treatment in both non-lactating and lactating cows is increased embryo growth rate such that the filamentous blastocyst is significantly longer in treated cows by day 17 (Bilby et al 2004, Bilby et al 2006b). These larger embryos produce more of the anti-luteolytic factor interferon-τ (IFN-τ, and accordingly, it may be IFN-τ that is locally influencing PPARδ transcription in endometrium. In vitro, IFN-τ increases PPARδ mRNA abundance in a bovine endometrial cell line (MacLaren et al 2006). Alternatively, another factor secreted by the embryo or induced by IFN-τ may influence PPARδ abundance locally. In the mouse and rat, PPARδ is induced in the uterine stroma at implantation sites, an effect dependent upon the blastocyst (Ding et al 2003a, Ding et al 2003b).

Western blot expression of PPARδ protein does not correlate with mRNA abundances in bovine endometrium. This is consistent with previous observations in the mouse in a number of tissues, including endometrium (Ding et al 2003b). Similarly, adipocytes express dramatically increasing abundances of PPARδ mRNA through the differentiation process yet have very similar expression of protein (Larsen et al 2002). Four alternative promoters with varying translation efficiencies have been identified in the murine PPARδ gene, and it is suggested that there is significant regulation of PPARδ protein expression through these alternative promoters (Larsen et al 2002).

PPARδ is expressed widely during development, and is associated with differentiation of neural, adipose, epidermal and placental tissues, in particular (Desvergne and Wahli 1999, Feige et al 2006, Fournier et al 2007). There are species differences in uterine expression patterns. Ding and coworkers (Ding et al 2003b) observed, in response to estrogen, PPARδ expression in murine subluminal stroma at implantation sites as well as in decidua and in glandular epi-

thelium, but not in the luminal epithelium. Localization patterns are similar in the mink (Desmarais et al 2002), but differ somewhat in the rat: epithelial expression declines from day 1 to 5 of pregnancy, then re-appears in both implantation-site epithelium and stroma following attachment, and appears as well in decidua (Ding et al 2003a). In contrast, we consistently observed expression of PPARδ protein in the luminal and glandular epithelia as well as subluminal stroma of the bovine uterus at day 17 of the estrous cycle or pregnancy. This distribution pattern is similar to those observed in sheep and pigs (Cammas et al 2006, Lord et al 2006).

The subtle but significant reduction in reactivity to PPARδ antibodies in the endometrial epithelia in response to fish oil supplementation is consistent with the observed reduction in PPARδ mRNA abundance. Recognizing that the EMSAs are not designed as quantitative assays, it is interesting that binding of endometrial nuclear protein extract to the PPARδ response element appeared to be increased by fish oil, while binding to the classic PPRE was not affected in the same way. While EPA and DHA are recognized as ligands of PPARs, the affinity for the PPAR varies with cell type and may also depend upon oxidation state of the long-chain n-3 PUFA (Lee and Hwang 2002, Sethi et al 2002).

While clear functions of PPARδ have not been identified, it is apparent that this PPAR affects differentiation of epithelial cells in particular, so its expression in the uterine luminal epithelium is not surprising. Another common feature of localization of PPARδ Discussion is its coincident expression in sites of prostaglandin synthesis and action. In the mouse, endometrial prostaglandin H synthase-2 (PGHS-2) induction of prostaglandin I_2 (PGI_2) is associated with activation of PPARδ, and the PPAR agonist carbaprostacyclin can restore implantation in PGHS-2 deficient mice (Lim et al 1999). In the mink, activated blastocysts produce PGI2, and either the presence of an active blastocyst or PGI2 increase both PPARδ mRNA expression and activation in a mink uterine cell line (Desmarais et al 2002). These observations are difficult to explain given recent evidence by Fauti and coworkers (Fauti et al 2006) that PGI_2 does not activate PPARδ in four cell lines, although estrogen agonists stimulated both PGI_2 and PGHS-2 synthesis in those studies. Estrogen also increases PPARδ in muscle cells (D'Eon et al 2005). It has been shown that activation of PPARδ results in enhanced expression of the prostaglandin EP4 receptor and response to prostaglandin E2 in human lung carcinoma cells (Han et al 2005). In bovine endometrium, expression of EP4 has not been detected, and historical radioligand binding studies indicate a limited ability to bind PGI_2 (Chegini and Rao 1989, Arosh et al 2003). The inducible PGHS-2 enzyme is expressed in bovine luminal epithelium (Emond et al 2004) confirming that, as in other species, there

is overlap in the distribution patterns of PGHS-2 and PPARδ. Our work suggests a complex relationship that is reflected in the myriad of treatment interactions observed by immunohistochemistry of this protein in the luminal epithelium. In vitro activation of PPARδ with the agonist carbaprostacyclin stimulates the accumulation of both $PGF_{2\alpha}$ and PGE_2, reversing the suppressive effect of IFN-τ (MacLaren et al 2006). The less specific PPARδ ligand EPA increases PPARδ mRNA abundances *in vitro*, but long term supplementation is associated with overall suppression of PGF_{2a} and PGE_2 accumulation *in vivo* and *in vitro* (Mattos et al 2002, MacLaren et al 2006).

PPAR alpha is present in bovine endometrium at day 17 post-estrus regardless of pregnancy status. This PPAR has also been observed in endometrium of the rat (Nunez et al 1998, Escher et al 2001), and more recently, the sheep (Cammas et al 2006). PPARα is associated with fatty acid oxidation, and is known to be expressed in tissues that are highly metabolically active. Several reported characteristics of this protein make it interesting in terms of bovine endometrial function. First, activation of this PPAR has been shown in other cell types to influence PGHS-2 expression, as well as expression of other cytokines, suggesting potential influence on prostaglandin synthesis (Kalajdzic et al 2002). Second, *in vitro* this PPAR can directly bind the estrogen response element and stimulate transcription (Nunez et al 1998) and recently estrogen acting through estrogen receptor-α has been shown to decrease PPARα mRNA abundance (Faddy et al 2006). Finally, growth hormone concentrations affect PPARα mRNA expression in liver (Carlsson et al 2001) and there is cross-talk between PPARα and growth hormone along the JAK-STAT pathway (Zhou and Waxman 1999). The interaction of bST with pregnancy status on endometrial PPARα mRNA abundance in lactating dairy cows of the present study is interesting given the beneficial effects of growth hormone treatment on pregnancy rates in lactating dairy cows. The JAK-STAT pathway is the pathway affected by IFN-τ the embryo-secreted pregnancy recognition factor in ruminants.

Although PPARα protein expression was similar in the endometrium of all cows, significant differences in mRNA abundances were observed among treatments. Most interestingly, the expression patterns associated with the treatments depended upon whether the cows were lactating or not. In the non-lactating Holstein cows, pregnancy and bST treatment at insemination or day of induced LH surge and 11 days later decreased steady state abundances of PPARα RNA. The decrease in mRNA in response to bST is consistent with prior studies in the liver showing that prolonged growth hormone administration reduces PPARα transcription (Carlsson et al 2001). In lactating Holsteins, bST did not affect PPARα mRNA in cyclic animals, but did increase PPARα mRNA abundance in pregnant an-

imals. The importance of lactation status in determining the bST response was described previously in our laboratory (Bilby et al 2004, Bilby et al 2006b). Nonlactating Holsteins develop supraphysiologic circulating concentrations of IGF-1 in response to bST, and do not show improved reproductive performance (Bilby et al 2006b, Thatcher et al 2006). The lactating Holstein cow has extremely low concentrations of IGF-1, and responds to bST administration with circulating concentrations of IGF-1 comparable to those found in untreated, non-lactating animals and improved pregnancy rates (Bilby et al 2004, Thatcher et al 2006). Altering lactation status thus provides an interesting model that confirms a relationship between the somatotropin axis and PPARα regulation. From the perspective of studying the mechanisms of pregnancy rate improvement in response to bST at insemination in lactating cows, the positive relationship between bST treatment, PPARα mRNA abundance and improved pregnancy rates warrants more investigation.

Electrophoretic shift mobility analyses suggest endometrial nuclear protein binding to both the PPRE and DRE. The PPRE is reported to bind heterodimers of either ligand-activated PPARα and RXR or PPARγ and RXR, with conflicting evidence of PPARδ -RXR binding (Desvergne and Wahli 1999, He et al 1999, Feige et al 2006). Given the low abundance of PPARγ mRNA in endometrium, we expect that the observed protein-PPRE complexes primarily reflect PPARα activation and heterodimer formation, although two shift complexes were observed in endometrium, compared with only one in kidney. Somewhat surprisingly, the EPA/DHA-rich fish oil supplement did not increase PPARα binding as indicated by the EMSA, since both EPA and DHA are considered ligands and activators of PPARα (Krey et al 1997). However, DHA-PPARα binding inhibits PPRE activation in some cell systems (Lee and Hwang 2002). It is also possible that the PPARs were de-phosphorylated in the extract and masked treatment effects to some extent, since a specific phosphatase inhibitor was not included in our extraction buffer. Pregnancy and bST treatment tended to increase the intensity of the resulting protein-PPRE complexes in lactating cows, although no endometrial treatment showed the extent of complex formation that occurred in kidney.

The PPARδ response element DRE has only been described to bind activated PPARδ heterodimers, not PPARα or PPARγ, and is not well characterized in terms of knowledge of the genes that carry this response element (He et al 1999). Consistent with what was observed for the PPRE drastic differences in protein-response element complex formation were not observed among endometrial samples from different treatments. However, as expected, fish oil feeding was associated with increased DRE binding. In addition, samples from bST treated animals showed increased DRE binding in cyclic but not pregnant

animals. Although not conclusive on their own, these observations support a role for PPARδ in the endometrial responses of omega-3 fatty acids and bST at the time of pregnancy recognition. There was an inverse relationship overall between relative activation and binding of the receptor and its mRNA level, suggesting that activation may reduce transcription or increase turnover of the PPARδ transcript.

Similar to what we observed in the current experiments, rat endometrium does not transcribe significant amounts of PPARγ mRNA (Escher et al 2001). We also did not detect PPARγ mRNA in a bovine endometrial cell line (MacLaren et al 2006), although this PPAR is readily detected by northern blotting in several bovine tissues, including ovary (Sundvold et al 1997). Its expression also has been reported in the endometrium of the sheep (Cammas et al 2006), although transcript abundances are low on days 12-14, which is physiologically comparable to the stage studied here in cattle, day 17. It is expressed in both cyclic and pregnant porcine endometrium (Lord et al 2006). PPARγ is known for its role in adipose and influences on lipid metabolism, and has also been detected in other reproductive tissues, including ovary, breast tissue and placenta (Cui et al 2002, Feige et al 2006). *In vitro*, it has been shown to bind the human ERE (Nunez et al 1998). Selective knockout of PPARγ function in the mouse ovary resulted in lower circulating concentrations of progesterone and implantation failure (Cui et al 2002). Regulation of PPARγ mRNA transcription in reproductive tissues has not been characterized, but EPA is known to increase mRNA abundance in adipocytes (Chambrier et al 2002). However, the current results indicate that PPARγ is not important for the endometrial response to omega-3 PUFA or bST in lactating dairy cows.

► Conclusions

In summary, treatment of lactating Holstein cows with bST at insemination and 11 days later is associated with increased endometrial abundances of PPARδ and PPARα mRNA on day 17 of pregnancy compared to the response of pregnant cows not treated with bST. Supplementation with the n-3 PUFA-rich fish oil decreases abundance of PPARδ mRNA but does not impact the abundance of PPARα mRNA. Impacts on protein expression are modest, although there is preliminary evidence that PPAR activation is affected by fish oil supplementation. The results are consistent with the hypothesis that PPARα and PPARδ are involved in the lactating cow response to management strategies that improve pregnancy rates such as bST treatment at breeding and supplementary n-3 PUFAs.

► Acknowledgements

tion, respectively. We also thank Dr. Hai Choo Smith for her patient and expert technical expertise in immunohistochemistry. Appreciation of donations is extended to Select Sires for semen, Pfizer Animal Health for Lutalyse, Intervet Inc. for Fertagyl, and Virtus Nutrition for EnerG II Reproduction formula for these experiments. This work was supported by the NRI Competitive Grant Program, USDA Grant 98-35203-6367, to WWT and an NSERC Discovery Grant to LAM.

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