The effect of a diet supplemented with the *n*-6 polyunsaturated fatty acid linoleic acid on prostaglandin production in early- and late-pregnant ewes

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Abstract

Polyunsaturated fatty acids derived from the diet are incorporated into cell membranes where they act as precursors for prostaglandin (PG) synthesis. Linoleic acid (LA; 18:2 *n*-6) is a major constituent of plant oils and its consumption in Westernized populations is increasing. This study investigated the influence of LA on PG production by the uterus and placenta. Pregnant ewes were fed a control or an LA-enriched diet. Oxytocin (OT) was injected on day 45 (early) or day 133 (late) of gestation to measure the release of 13,14-dihydro-15-keto PGF_{2α} (PGFM). Ewes were killed on day 46 or day 138 for collection of uterine intercaruncular endometrium and fetal allantochorion. Basal and stimulated PG release from explant cultures was assessed before and after *in vitro* treatment with OT, lipopolysaccharide (LPS), dexamethasone (DEX) or calcium ionophore (CaI). Expression of cyclooxygenase (COX)-1 and COX-2 was determined by Western blot in endometrium of late-gestation ewes. Circulating PGFM levels *in vivo* did not differ according to diet but there were highly significant differences in the release of PGs *in vitro*. Basal production of PGF_{2α} and

PGE₂ by the endometrium and of PGE₂ by the allanto-chorion were all higher in tissues from LA-supplemented ewes. Endometrial tissues produced more PG following OT and CaI treatment, whereas DEX inhibited production of both PGs at both stages of gestation. In allanto-chorion collected at day 46 LPS did not significantly alter PGE₂ release and DEX increased output, whereas at day 138 LPS was stimulatory but DEX was inhibitory. These data show that a high-LA diet can significantly increase the ability of both endometrium and placental tissues to produce PGs *in vitro*. This effect of diet may only become apparent after a sustained period of PG release, so was not seen following the brief pulse caused by OT treatment *in vivo*. As COX protein levels were unaltered, the main influence was likely to be via conversion of LA to arachidonic acid, providing an increased supply of precursor. These results support previous studies which suggest that alterations in dietary polyunsaturated fatty acids may influence the time of labour.

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Introduction

Dietary polyunsaturated fatty acids (PUFAs) provide the body with energy, contribute structural components to cell membranes and also act as the precursors for prostaglandin (PG) synthesis. Linoleic acid (LA; 18:2 *n*-6) is the major fatty acid component in plant oils such as corn, sunflower, rapeseed and soybean oils (Sargent 1977) and is thus a common constituent of both human and animal diets. LA can be metabolized within the body to arachidonic acid (AA; 20:4 *n*-6; Kinsella *et al.* 1990, Bezard *et al.* 1994). Two series of PGs, including PGF_{2α} and PGE₂, are derived from AA following its release from phospholipid membrane stores by the action of the enzyme phospholipase A₂ (PLA₂). Free AA is

then metabolized to PGF_{2α} and PGE₂ through the action of the cyclooxygenase (COX) enzymes COX-1 and COX-2 (Smith *et al.* 1991). COX-1 is generally considered to be a housekeeping gene and responsible for immediate responses whereas COX-2 is inducible by a variety of agents and is crucial for more-sustained PG generation (Smith *et al.* 1996, Murakami & Kudo 2004). There is, however, evidence showing that COX-1 can be upregulated in response to angiogenic factors (e.g. Sales *et al.* 2002) and during cellular differentiation (Murakami & Kudo 2004). Experiments using both gene-deletion and pharmacological treatments with specific COX-1 and COX-2 inhibitors have shown that parturition in mice is normally dependent on upregulation of COX-1 in the endometrium

(Gross *et al.* 1998, Reese *et al.* 2000, Loftin *et al.* 2002). In the ewe, however, COX-2 expression in the endometrium is increased during both spontaneous and dexamethasone (DEX)-induced labour, whereas there is no change in COX-1 expression at this time (Zhang *et al.* 1996, Gibb *et al.* 2000, McClaren *et al.* 2000). In the uterus and placenta PG production and release can be modulated by a variety of physiological and pharmacological stimuli, for example oxytocin (OT; Lee and Silvia 1994), lipopolysaccharide (LPS; Flynn & Hoff 1995), calcium ionophore (CaI; Poyser 1987) and glucocorticoids (Whittle *et al.* 2001). Manipulation of the dietary PUFA content can also influence the production of PGs. Following increased human consumption of *n*-6 fatty acids, eicosanoid metabolic products from AA, specifically PGs and thromboxanes, are formed in larger quantities than when the diet is high in *n*-3 fatty acids (Simopoulos 2002).

However, not all reports on the effects of *n*-6 PUFA on PG synthesis are consistent. Some evidence suggests that LA enhances PG production by providing more precursor for conversion to AA (e.g. Connolly *et al.* 1996, Nakaya *et al.* 2001). Other studies have found an inhibitory effect. Both LA and *n*-3 PUFAs may suppress PG production at a number of different stages of the biosynthetic pathway. These include (i) inhibition of AA synthesis during the desaturation and elongation processes in the liver (Bezard *et al.* 1994), (ii) *n*-3 competing with *n*-6 PUFAs, especially AA, for incorporation into cell-membrane phospholipids, decreasing the available AA for COX (German *et al.* 1988) and (iii) direct blocking of COX activity (Staples *et al.* 1998, Marnett *et al.* 1999). In ruminants the effects of PUFAs have been investigated with a view to modulating uterine PG production during luteolysis or in early pregnancy (Staples *et al.* 1998, Abayasekara & Wathes 1999). PGs also play a critical role in many aspects of parturition (Thorburn & Challis 1979, Challis *et al.* 1997) and there is considerable evidence that dietary PUFAs can influence the timing of labour. For example, parturition is delayed in *n*-6 PUFA-deficient rats (Leaver *et al.* 1986), and women who delivered early were found to have a raised pool of *n*-6 PUFAs in plasma (Allen & Harris 2001). On the other hand, women with an increased *n*-3 PUFA intake had increased pregnancy duration (Olsen *et al.* 1992). The literature thus shows that manipulation of dietary PUFAs can influence PG production, but the end result is hard to predict. This study investigated the effect of increased *n*-6 PUFA supplementation on PG production during pregnancy by feeding ewes a diet enriched with LA. PG production in control and LA-supplemented ewes was compared: (a) in response to an *in vivo* OT challenge and (b) *in vitro* in cultured explants of endometrium and allantochorion. The experiment was repeated at two stages of gestation, early (day 45) and late (day 133).

Materials and Methods

Animals

All experimental work was performed under the Animals (Scientific Procedures) Act 1986. Mature Welsh mountain ewes had oestrous cycles synchronized by insertion of an intravaginal progestagen sponge (Upjohn, Crawley, West Sussex, UK) for 14 days. Immediately after sponge removal, 125 µg synthetic PG (Estrumate, Schering-Plough Animal Health, Welwyn Garden City, Herts, UK) was administered i.m. and the ewes were housed with a raddled ram for 48–96 h after sponge removal. Pregnancies were confirmed initially by measurement of plasma progesterone concentrations 16 days after mating. In the late-gestation group this was followed by an ultrasound scan at mid-gestation. The mean gestation length in a similar group of untreated ewes was 145 ± 0.4 days ($n=7$). Two experiments were performed. In both cases ewes were individually housed prior to the start of the experimental protocol and were gradually introduced to the experimental diet over a 7-day period. In experiment 1 (early gestation), ewes were placed on a control ($n=5$) or LA-supplemented (LA diet; $n=6$) diet from 7 days prior to the start of oestrous synchronization until euthanasia at 46 days gestation. In experiment 2 (late gestation) ewes were placed on a control ($n=16$) or LA-supplemented ($n=16$) diet from 96 days of gestation until euthanasia at 138 days of gestation.

Diets

Diets were calculated on an individual ewe basis (reviewed every week) to meet the maintenance requirement of the ewe according to her weight and stage of gestation and were mixed individually and fed each morning. The *n*-6 PUFA was fed as SoyPreme (Boregaard UK, Warrington, UK), a heat-treated product of xylose and cracked soya-bean. This reaction reduces the degradability of the protein and protects the PUFAs from biohydrogenation in the rumen (Abel-Caines *et al.* 1998). Following such treatment approximately 55% of the dietary lipid bypasses the rumen, whereas for untreated feed this figure is only about 15%. Each diet was based on a mixture of maize silage, grass silage and concentrates with added SoyPreme on the LA-supplemented diet only. The diets were formulated to be isonitrogenous and isoenergetic. Details of dietary analyses and PUFA contents (ADAS Laboratories, Wolverhampton, UK) are given in Tables 1 and 2 respectively. Experiments 1 and 2 were performed in different years, so there were minor differences in dietary composition. Based on these figures it was estimated that, in experiment 1, 1.3 g/day *n*-6 PUFA would reach the small intestine in control-fed ewes compared with 8.5 g/day in the LA-supplemented ewes. In experiment 2, where dietary intake was higher later in gestation,

Table 1 Sheep diets. The amounts fed are based on wet weight for ewes in experiment 1 at day 45 of gestation (mean ewe weight, 47 kg) and in experiment 2 at day 135 of gestation (mean ewe weight, 60 kg). The actual amounts fed were adjusted according to ewe weight, which increased during pregnancy

(a)

Ingredient	Diet (g/sheep per day)			
	Control diet		LA diet	
	Day 45	Day 135	Day 45	Day 135
Grass silage	893	1390	893	1465
Maize silage	833	1390	833	1465
Wheat	95	160	85	96
Sugar beet pulp	50	106	60	48
Rapeseed meal	75	106	60	48
Soypass	65	160	—	0
Megalac	32.5	20	15	4
SoyPreme	—	—	140	217
Maize gluten meal	—	—	—	96
Total	2043	3332	2086	3439

(b)

	Feed analysis (mixed ration)			
	Dry matter	As fed	Dry matter	As fed
Day 45				
DM (%)		43.4		46.7
Total crude protein (%)	14.5	6.3	14.1	6.6
NCGD (%)	62.0		65.9	
Oil (acid-hydrolysed) (%)	4.6	2.0	5.8	2.7
Total ash (%)	7.1	3.1	6.5	3.0
ME (MJ/kg DM)		10.3		10.7
Day 135				
DM (%)		29.7		28.9
Total crude protein (%)	13.3	4.0	13.6	3.9
NCGD (%)	62.6		62.9	
Oil (acid-hydrolysed) (%)	3.8	1.9	4.5	2.6
Total ash (%)	7.6	2.2	7.6	2.2
ME (MJ/kg DM)		9.7		9.9

DM, dry matter; NCGD, neutral cellulase gammanse digestibility; ME, metabolizable energy.

the equivalent figures were 2.5 and 13.7 g/day. The LA-supplemented diet therefore increased the LA consumption approximately 6-fold.

OT challenge

When pregnant sheep reached day 44 (experiment 1) or 132 (experiment 2) of gestation a jugular-vein cannula was fitted. The following day, 5 ml blood samples were taken 60, 45, 30, 15 and 0 min before and 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min after administration of 10 IU OT given via the jugular-vein cannula (Oxytocin-s; Intervet UK, Milton Keynes, Bucks, UK). The blood samples were put into heparinized tubes, placed on ice and centrifuged

Table 2 PUFA analysis of feed. Results are given as percentages of total fatty acids in the lipid component; all other fatty acids were not detectable (<1%)

(a)

Fatty acid	Proportion of total fatty acids (%)		
	Control diet	LA diet	Neat SoyPreme
C16:0	13	12	11
C18:0	3	3	4
C18:1(<i>n</i> -7) <i>cis</i>	2	2	1
C18:1(<i>n</i> -9) <i>cis</i>	13	16	21
C18:2(<i>n</i> -6) <i>cis</i>	29	41	53
C18:3(<i>n</i> -3) <i>cis</i>	22	16	8

at 1600 g at 4 °C. The resulting plasma was frozen and stored at -20 °C. The stable metabolite of PGF_{2α}, 13,14-dihydro-15-keto PGF_{2α} (PGFM) was then quantified at each time point by RIA.

Explant culture

Cell-culture reagents were from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Ewes were killed by captive bolt on day 46 (all ewes in experiment 1) or day 138 of gestation (eight ewes per diet from experiment 2). Reproductive tracts were placed in a laminar flow hood to maintain sterility. The uterus was opened and the fetus was removed. Maternal intercaruncular (IC) endometrium was separated from fetal allantochorion by manual dissection. Strips of each tissue were placed in basic Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Gibco Life Technologies, Paisley, UK) containing 1.125 g/l fatty acid-free BSA and antibiotics (50 000 units/l penicillin and 50 mg/l streptomycin). The method for explant culture has been described previously (Leung *et al.* 2001). Strips of IC and allantochorion tissue were chopped into 1 mm³ cubes. Tissue samples were blotted dry and weighed to provide 150 mg/dish. This was placed on a lens tissue on a stainless steel grid in a petri dish (30 mm×30 mm×0.5 mm) containing 6 ml basic medium supplemented with ITS media supplement to provide a final concentration of 0.5 µg/ml insulin, 0.5 µg/ml transferrin and 0.5 ng/ml selenium. The tissue explants were then cultured at 39 °C with 5% CO₂ for 42 h in a humidified incubator with the medium changed after 24 h. At the end of the 42 h culture period the explants were challenged for 24 h with (i) control medium, (ii) OT (250 nM), (iii) DEX (5 µM), (iv) CaI (A23187; 10 µM) or (v) LPS (*Escherichia coli* 026:B6; 0.1 µg/ml). OT was only tested on maternal endometrial tissues whereas LPS was used on fetal allantochorion (which lacks OT receptors). Medium was collected at 2 and 24 h after the start of the challenge. The rationale behind the times chosen for medium collection for PG measurement was as follows. The first 24 h period was discarded as this represented release from damaged tissue following the chopping procedure. The next 18 h provided a period to assess basal PG production. The challenges were performed after 42 h as we have previously shown that this is the time needed to get upregulation of OT receptors *in vitro*, and we wished to challenge with OT (Leung & Wathes 2000). Following challenge, we investigated both the immediate (2 h) and longer-term (24 h) responses as the different ligands used were known to act over differing time courses in terms of influencing PG synthesis. Each treatment was performed in quadruplicate or triplicate. The culture medium was frozen and stored at -20 °C for subsequent measurement of PGF_{2α} and PGE₂ by RIA. Both PGs were measured for the IC explants but, as PGE₂ is the main product of

allantochorion, only this was measured in the allantochorion samples.

Treatment preparation

OT powder was dissolved in sterile distilled water containing 0.01% acetic acid. The reconstituted stock OT was diluted further with DMEM/Ham's F12 basic medium to give a final concentration of 250 nM. DEX was dissolved in 100% ethanol at 20 mM and diluted with basic medium to a final concentration of 5 µM. The CaI A23187 was dissolved in 100% ethanol to a stock solution of 10 mM. This was diluted with basic medium to a final concentration of 10 µM. LPS was dissolved in distilled water at 500 µg/ml and diluted in basic culture medium to provide a final concentration of 0.1 µg/ml. The final ethanol concentration in the cultures did not exceed 0.1%.

PG RIAs

PGFM in the blood samples was measured as described previously (Robinson *et al.* 2002). The tracer (13,14-dihydro-15-keto-[5,6,8,9,11,12,14(*n*)-³H]PGF_{2α}) was from Amersham International (Little Chalfont, Bucks, UK), the standards were supplied by Sigma-Aldrich and the antiserum was a kind gift from Dr H. Kindahl (Dept. of Obstetrics and Gynaecology, Swedish University of Agricultural Science, Uppsala, Sweden). The limit of detection was 1 pg/tube. The intra-assay and inter-assay coefficients of variation were 7.6 and 14.3% respectively.

PGE₂ and PGF_{2α} in the media samples were quantified as described previously following dilution in 0.05 M Tris buffer containing 0.1% gelatin and 0.01% sodium azide (Cheng *et al.* 2003). The tritiated tracers of PGE₂ ([5,6,8,11,12,14,15(*n*)-³H]PGE₂) and PGF_{2α} ([5,6,8,9,11,12,14,15(*n*)-³H]PGF_{2α}) were from Amersham International and the standards of PGE₂ and PGF_{2α} were from Sigma-Aldrich. The antisera to these two PGs were a kind gift from Dr N.L. Poyser (University of Edinburgh, Edinburgh, UK; Poyser 1987). Their cross-reactivities were as follows: PGF_{2α} antiserum, 34% with PGF_{1α} and 25% with PGF_{3α}; PGE₂ antiserum, 23% with PGE₁ and 15% with PGE₃. The limit of detection was 1 pg/tube for PGF_{2α} and 2 pg/tube for PGE₂. The intra-assay and inter-assay coefficients of variation were 3.5 and 6.3% for PGE₂, and 4.1 and 9.6% for PGF_{2α} respectively.

Preparation of tissue lysates

In experiment 2, additional strips of IC were collected at slaughter, placed in cryovials, frozen immediately on dry ice and stored at -80 °C. Tissues (1 g) were homogenized in 10 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA, 250 mM sucrose and protease-inhibitor tablets (Protease inhibitor cocktail tablets, one dissolved per 50 ml buffer; Roche catalogue no. 1 836 145;

Table 3 Effects of the control or LA-supplemented diet on basal and peak concentrations of PGFM (mean $\pm$ s.e.m.) before and after an OT challenge. Basal values were obtained from five samples collected at 15 min intervals before the OT challenge. Peak concentration was the highest value reached in the 2 h sampling period after the OT challenge

	PGFM concentration (ng/ml)			
	Day 45		Day 133	
	Control (n=5)	LA (n=6)	Control (n=14)	LA (n=14)
Basal	0.06 $\pm$ 0.004 ^b	0.08 $\pm$ 0.007 ^b	0.18 $\pm$ 0.01 ^a	0.19 $\pm$ 0.01 ^a
Peak	0.77 $\pm$ 0.14	0.85 $\pm$ 0.14	0.75 $\pm$ 0.07	0.74 $\pm$ 0.06

No differences between diets were significant, but basal values were higher at day 133 than at day 45 (^{a>b}, $P<0.001$).

F. Hoffman La Roche, Basel, Switzerland). The homogenate was then centrifuged at 800 *g* for 10 min at 4 °C, and the supernatant was collected and ultracentrifuged at 100 000 *g* for 60 min at 4 °C to obtain the microsomal fractions. Microsomal preparations were then solubilized in homogenization buffer containing 100 mM phenylmethanesulfonyl fluoride (PMSF) and stored at –80 °C. The protein content was determined by BCA protein assay kit (Sigma–Aldrich).

SDS/PAGE and Western immunoblotting for COX

Tissue lysate proteins were separated using 10% SDS/PAGE (Wheeler-Jones *et al.* 1996). Following electrophoresis, proteins were transferred to Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore UK, Watford, UK) using the Bio-Rad semi-dry blotting system. After transfer of proteins to a PVDF membrane, non-specific sites were blocked using a solution of Tris-buffered saline with Tween (TBS-T; 50 mM Tris, 150 mM NaCl, 0.02% (v/v) Tween-20, pH 7.4) containing 10% (w/v) BSA, for 2 h at room temperature with gentle agitation. The membrane was then incubated with either anti-COX-1 antibody (Cayman Chemical Company, Ann Arbor, MI, USA) at 1:500 dilution or anti-COX-2 antibody (Santa Cruz Biotechnology, Calne, Wilts, UK) at 1:1000 dilution in TBS-T containing 10% (w/v) BSA overnight at 4 °C at the maximum agitation speed. The membrane was then washed for 6 $\times$ 10 min in TBS-T. Following the incubation of the membrane in a 1:10 000 dilution (in TBS-T plus 0.2% (w/v) BSA) of horseradish peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG (both from Pierce, Chester, UK) for 1 h, the membrane was washed a further eight times for 10 min in TBS-T. Immunoreactive proteins were visualized using enhanced chemiluminescence (ECL; Amersham).

Blots were also assessed for β -actin expression using anti- β -actin antibody (Abcam, Cambridge, UK) and horseradish peroxidase-conjugated goat anti-mouse IgG (Transduction Laboratories, Cowley, Oxford, UK).

Densitometric quantification of immunoreactive bands was carried out using a Bio-Rad model GS-690 imaging densitometer with Bio-Rad Quantity One software, version 4.4.0 (Bio-Rad Laboratories, Hercules, CA, USA).

Data analysis

The values are quoted as means $\pm$ s.e.m. Analysis of experimental data in this study was performed on the basis of the two dietary groups, control and LA-supplemented. All data were checked for homogeneity and if not homogeneous data were either log- or square-root-transformed to achieve a normal distribution. Statistical analysis for the PGFM concentrations following *in vivo* OT challenge was carried out using a repeated-measures analysis via Proc Mixed built in SAS 8.0 (SAS Institute, Cary, NC, USA). Comparisons of PGF_{2 α} and PGE₂ production by placental tissue explants were carried out using analysis of variance via general linear and mixed linear models in SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). Where significant difference was achieved ($P<0.05$), least-square means based on Fisher's least significant difference (LSD) for multiple comparisons were calculated to examine the differences between the fixed effects. Optical density units from the Western blot analysis were analysed with an unpaired *t*-test using GraphPad Prism version 2.01 (GraphPad Software, San Diego, CA, USA).

Results

OT challenge

Basal and peak levels of plasma PGFM were measured in ewes on the two diets at 45 and 133 days gestation in plasma samples taken for 1 h before until 2 h following the OT challenge. Basal PGFM concentrations were higher at day 133 than at day 45, but peak values were similar (Table 3). There were no significant differences in either basal or peak levels of PGFM between the control and

Figure 1 Effect of a bolus treatment with 10 IU OT (given at time 0) on maternal plasma PGFM on (a) day 45 of gestation and (b) day 133 of gestation in ewes fed a control or LA-supplemented diet. Values are given as means $\pm$ S.E.M. for control (○) ($n=5$) and LA-supplemented (■) ($n=6$) ewes at day 45 and for control (○) ($n=14$) and LA-supplemented (■) ($n=14$) ewes at day 133.

LA-supplemented diets at either time point and the pattern of release of PGFM into the maternal blood plasma in response to OT was not influenced by the experimental diet (Fig. 1).

Explant cultures: basal production of PGs

Basal concentrations of PGs were estimated by measurement in medium over the 18-h period from 24 to 42 h following establishment of the explant cultures (Table 4). In experiment 1 (early gestation) the $\text{PGF}_{2\alpha}$ and PGE_2 production by IC explants was increased in tissue obtained from ewes on the LA-supplemented diet. Synthesis of PGE_2 by the fetal allantochorion was also significantly increased by dietary $n-6$ PUFA. Similar results were obtained for experiment 2 (late gestation), when all tested PGs were again significantly increased by the LA-supplemented diet. Whereas PG production by the en-

dometrium was of a similar order of magnitude in tissues obtained at both stages of gestation, production of PGE_2 by the allantochorion increased over 30-fold between 46 and 138 days.

Explant cultures: stimulated production of PGs

Following the initial 42 h culture, the tissue explants were challenged *in vitro*. The effects of treatment on $\text{PGE}_{2\alpha}$ secretion by maternal IC endometrium are illustrated in Fig. 2. The OT and CaI treatments stimulated $\text{PGE}_{2\alpha}$ output at both 2 and 24 h from tissue obtained in both early and late gestation. DEX had no effect at 2 h but caused a significant inhibition after 24 h in the early gestation tissue. Tissue obtained from ewes on the LA-supplemented diet produced consistently more $\text{PGE}_{2\alpha}$ at 2 h than the tissue obtained from control-fed ewes and subsequently treated similarly *in vitro*. The effect of the LA diet was less profound after 24 h; significant increase in $\text{PGE}_{2\alpha}$ production was observed only in the tissue challenged with DEX in the early-pregnancy ewes and following control-medium and CaI treatment in the late-pregnancy ewes.

PGE_2 was only measured in the IC tissue collected in late gestation (Fig. 3). The effects of the challenge treatments were similar to those reported for $\text{PGE}_{2\alpha}$. OT stimulated PGE_2 at 24 h, CaI treatment stimulated PGE_2 output at both 2 and 24 h and DEX had no effect at 2 h but caused a significant inhibition after 24 h. Tissue from the LA-supplemented ewes produced more PGE_2 following control-medium, OT and DEX treatments at 2 h and following CaI treatments at 24 h.

PGE_2 production was assessed in explants of fetal allantochorion collected from both early- and late-gestation ewes (Fig. 4). In these cultures LPS rather than OT was used as a potential modulator of PG production. The effects of the challenges were somewhat different to those observed in the maternal IC tissue, possibly associated with the large overall increase in the ability of the tissue to produce PGE_2 at the later time period. After 2 h treatment of the early-pregnancy tissue none of the *in vitro* treatments had any effect. After 24 h of treatment, PGE_2 production was enhanced by DEX but inhibited by CaI. In the late-pregnancy tissue, none of the challenge treatments influenced PGE_2 output after 2 h. After 24 h, PGE_2 output was unaffected by DEX and increased by LPS and CaI. In relation to the diet, LA supplementation *in vivo* increased PGE_2 output at both time points regardless of treatment *in vitro* for the tissue collected in early pregnancy. In the late-pregnancy tissue, there was again a significant increase following all challenges at 2 h and after the control challenge at 24 h.

COX expression

COX-1 and COX-2 protein concentrations were measured in tissue from late-pregnancy intercotyledonary

Table 4 Effects of a control or LA-supplemented diet on basal PGF_{2α} and PGF₂ release (mean ± S.E.M., measured in ng/ml per h) from cultured explants of maternal intercaruncular (IC) endometrium or fetal allantochorion (AC). Medium was analysed after an 18 h incubation period. There were 12 or 16 replicate dishes for each sheep

	Diet	No. of ewes	PG release (ng/ml per h)		
			IC		AC
			PGF _{2α}	PGE ₂	PGE ₂
Gestation stage					
Day 46	Control	5	111 ± 4.2	92 ± 6.2	1.9 ± 0.11
	LA	6	131 ± 5.8**	121 ± 12.3*	2.5 ± 0.16**
Day 138	Control	8	71 ± 4.1	61 ± 3.6	69 ± 3.7
	LA	8	144 ± 6.8**	87 ± 5.8**	89 ± 5.3**

Differences between the Control and LA diets at each time point are indicated. * $P < 0.05$ and ** $P < 0.01$.

endometrium by Western blot analysis. There was no detectable effect of the diet on COX expression (Fig. 5).

Discussion

Few previous studies have investigated the effect of a high *n*-6 PUFA diet on PG production during pregnancy, yet PGs play central roles in both fetal maturation and parturition (Liggins & Thorburn 1994). Westernized populations are currently increasing their intakes of vegetable oils containing high concentrations of PUFAs, in particular LA (18:2 *n*-6; Department of Health 1995). Within the UK, 2.5% of the female population consume over 20 g *n*-6 PUFA daily, mostly as LA (Office of Population Censuses and Surveys 1990). We show here that placental tissues from ewes fed a diet containing a 6-fold increase in LA consistently produced significantly more PG *in vitro* than tissues from ewes on the control diet.

In general, diets with a high AA content will increase phospholipid levels of AA and also increase PG production (German *et al.* 1996, Sinclair & Mann 1996). When diets contain *n*-6 PUFAs from further up the *n*-6 pathway, results are more variable (see Introduction). LA is converted to AA by position-specific $\Delta 6$ - and $\Delta 5$ -desaturase and CoA-dependent chain elongases, located primarily in the liver (Bezard *et al.* 1994, Sprecher 2000). This will lead to an increased AA component of plasma lipids and greater incorporation of AA into the phospholipid membranes of tissues which can subsequently be released for PG synthesis. This is supported by *in vitro* studies of ovine endometrium (Cheng *et al.* 2004) and human decidual cells (Arntzen *et al.* 1998), which both showed enhanced PG production in response to the *n*-6 PUFAs AA and dihomo- γ -linolenic acid (DGLA) supplemented *in vitro*. In related studies we have shown that a similar LA-supplemented diet to that used here increased plasma AA concentrations (Elmes *et al.* 2004) and was associated with earlier delivery in DEX-induced late-gestation ewes (Elmes *et al.* 2003).

Women giving birth to preterm infants were also reported to have a higher AA concentration in their blood than those delivering at normal term (Reece *et al.* 1997, Araya *et al.* 1998).

Our data suggest that the stimulatory effect of dietary LA on utero-placental PG production was more likely due to increased precursor availability than to upregulation of COX, as neither COX-1 nor COX-2 levels were increased in tissues obtained from the LA-supplemented ewes. However COX expression was not assessed following the explant culture period so it is possible that COX expression may have been further upregulated *in vitro* in tissues obtained from ewes on the high-LA diet. In support of this suggestion there is evidence that AA can itself upregulate COX-2 expression (Parent *et al.* 2003). PG production by particular tissues can also be influenced by the extent to which either COX-1 or COX-2 colocalize with particular terminal PG synthases in different sub-cellular compartments and the fact that COX-1 requires a greater supply of AA than COX-2 to be functional (Murakami & Kudo 2004). As the diet used in this study increased the supply of AA to the uterine tissues, activation of existing COX-1 could represent another potential mechanism whereby PG production could rise.

The *in vitro* study of stimulated production of PGs also produced interesting results, notably the different responses of the maternal and fetal tissues to DEX. The production of PGs by maternal endometrium was inhibited when challenged with this synthetic glucocorticoid, whereas in the fetal allantochorion PG secretion was stimulated in allantochorion tissues collected on day 46. In allantochorion obtained later in gestation (day 138) DEX caused slight, but non-significant, inhibition. An increase in fetal cortisol production is a key element in the signalling pathway which leads to parturition in sheep via induction of placental enzymes that redirect steroidogenic pathways to favour the production of oestrogen at the expense of progesterone (Anderson *et al.* 1975, Steele *et al.* 1976). This change in progesterone/oestrogen ratio

Figure 2 PGF_{2α} production by intercaruncular endometrium from ewes fed a control diet (open bars) or LA-supplemented diet (closed bars) following *in vitro* challenges with control medium (CM), 250 nM OT, 5 μM DEX or 10 μM Cal. The experiment was repeated at day 46 of gestation (5 control and 6 LA-supplemented ewes) and day 138 of gestation (8 control and 8 LA-supplemented ewes). The medium was collected at 2 and 24 h. Each treatment was replicated three or four times. Values are means ± S.E.M. Asterisks indicate significant differences between tissues from ewes on the control and LA-supplemented diets given the same treatment *in vitro*, **P*<0.05, ***P*<0.01. Bars with letters indicate significant differences due to the *in vitro* treatment, a<b<c, *P*<0.01.

Figure 3 PGE₂ production by intercaruncular endometrium obtained at day 138 of gestation from ewes fed a control ($n=8$) or LA-supplemented ($n=8$) diet following *in vitro* challenges with control medium (CM), 250 nM OT, 5 μ M DEX or 10 μ M Cal. The medium was collected at 2 and 24 h. Each treatment was replicated three or four times. Values are means $\pm$ S.E.M. Asterisks indicate significant differences between tissues from ewes on the control and LA-supplemented diets given the same treatment *in vitro*, * $P<0.05$, ** $P<0.01$. Bars with letters indicate significant differences due to the *in vitro* treatment: in (a) $a<b$, $P<0.05$; in (b) $a<b<c$, $P<0.01$.

activates COX-2, ultimately leading to an increase in PG production (McClaren *et al.* 1996, 2000). However, Whittle *et al.* (2000) showed that infusion of cortisol into the fetus beginning on day 125 of pregnancy increased both expression of COX-2 mRNA in placental trophoblast tissue and levels of fetal plasma PGE₂, even in the presence of the aromatase inhibitor 4-OHA. This suggested that a preceding increased output of placental oestrogen was not essential in the DEX-induced upregulation of placental PG production. On the other hand, DEX suppresses PG production from many tissues via its anti-inflammatory action. Glucocorticoids can inhibit PLA₂ activity by a variety of mechanisms, thus reducing precursor AA availability for the biosynthesis of PGs (Bailey 1991). Maternal endometrial COX-2 mRNA and protein concentrations only rose in the presence of increases in both fetal plasma cortisol and placental oestradiol production (Whittle *et al.* 2000). Whittle *et al.* (2000, 2001) therefore proposed that there are two separate pathways of intrauterine PG production. The first is a cortisol-dependent/oestradiol-independent pathway within fetal tissue and the other an oestradiol-dependent

pathway within maternal intrauterine tissues. Our data thus link well to this hypothesis, although they also suggest changes in responsiveness of the fetal allantochorion with stage of gestation.

Maternal explants challenged with OT significantly increased the production of PGF_{2 α} . This result was as expected based on many previous studies showing that OT can increase PG output via activation of cytosolic PLA₂ and COX-2 (Flint *et al.* 1986, Lee & Silvia 1994, Asselin *et al.* 1997). Cal acts by increasing intracellular calcium concentrations, another step in the OT signalling pathway (LaFrance & Goff 1990, Danet-Desnoyers *et al.* 1995). In maternal tissues PGF_{2 α} was consistently higher at both 2 and 24 h following Cal treatment. In the fetal allantochorion the time course of the PGE₂ response to Cal differed between early pregnancy (decrease at 24 h only) and late pregnancy (increase at 24 h only). There is no obvious reason for this discrepancy.

Fetal explants were challenged with LPS instead of OT as the allantochorion has no OT receptors (Wathes *et al.* 1996). LPS is a bacterial endotoxin that causes inflammation and fever and which increases PG production by

Figure 4 PGE₂ production by fetal allantochorion from ewes fed a control or LA-supplemented diet following *in vitro* challenges with control medium (CM), 0.1 µg/ml LPS, 5 µM DEX or 10 µM Cal. The experiment was repeated at day 46 of gestation (5 control and 6 LA-supplemented ewes) and day 138 of gestation (8 control and 8 LA-supplemented ewes). The medium was collected at 2 and 24 h. Each treatment was replicated three or four times. Values are means ± S.E.M. Asterisks indicate significant differences between tissues from ewes on the control and LA-supplemented diets given the same treatment *in vitro*, **P*<0.05, ***P*<0.01. Bars with letters indicate significant differences due to the *in vitro* treatment, a<b<c, *P*<0.01.

Figure 5 Western immunoblot data show COX expression in intercaruncular endometrium from ewes on day 138 of gestation. The data are representative of results from three (of five) animals for control and three (of seven) animals for LA-supplemented ewes. Densitometric analysis of each COX-1 or COX-2 band relative to the corresponding β -actin band has been pooled and is expressed as the mean $\pm$ S.E.M. in arbitrary optical density units. There was no difference in COX expression due to diet.

stimulation of PLA_2 release (Flynn & Hoff 1995). LPS also utilizes the Toll-like receptor 4 (Tlr4) to induce the transcription and expression of COX-2 (Poltorak *et al.* 1998, Rhee & Hwang 2000). Mitchell *et al.* (1991) reported a differential dose-response of human amnion and decidual cells to low-dose bacterial products, which stimulated PG production, and high doses, which were inhibitory. This upregulation of PG synthesis is widely attributed to being part of the mechanism by which uterine infection can cause preterm labour (Romero *et al.* 1989, Goldenberg *et al.* 2000). In our results LPS clearly stimulated PGE_2 release in the allantochorion collected in late gestation but had no significant effect on PGE_2 release in tissue collected in early pregnancy. This interesting result might perhaps be explained by alterations in Tlr4 signalling at different stages of gestation. In our experi-

ments this effect of LPS was not yet apparent after 2 h, so the time course of response was slower than that for OT.

The clear *in vitro* effect of dietary LA supplementation on enhanced production of two-series PGs was not, however, apparent by measurement of PGFM following the OT challenge *in vivo*. Development of uterine OT receptors is inhibited by interferon τ production in early pregnancy (Roberts *et al.* 1992, Wathes and Lamming 1995), but OT receptors subsequently appear from day 22 until term (Wathes *et al.* 1996). There is a peak in OT receptor expression in the placentome capsule at day 45 of gestation (Leung *et al.* 1998), coincident with the onset of regular uterine contractility (Garcia-Villar *et al.* 1984). Pregnant ewes continue to release $\text{PGF}_{2\alpha}$ in response to bolus OT administration during early pregnancy (Burgess *et al.* 1990), with a marked increase in response between days 25 and 65 and a further increase on day 145, near term (Meier *et al.* 1995). Our results showed that the PGFM production in response to a bolus injection of OT at either day 45 or 133 of gestation was not affected by feeding a diet high in LA. Previous work in the non-pregnant cow has produced conflicting results. Oldick *et al.* (1997) found that a fat source high in LA caused attenuation in OT-induced PGFM production in the peripheral plasma compared with control animals on day 15. Robinson *et al.* (2000) fed cows a similar SoyPreme diet to that used here and challenged with OT on days 15, 16 and 17 of the oestrous cycle. In this experiment the diet was found to have no effect on days 15 and 16, but induced a significant increase in PGFM release in SoyPreme fed cows on day 17.

With three studies on PGFM release in response to OT in LA-supplemented ruminants all having different outcomes, it is hard to interpret the results. The experimental diets used did have some differences in their PUFA contents, which may have been reflected in uterine phospholipid stores. Meier *et al.* (1997) also reported cyclical variation in ovine endometrial phospholipid concentrations, which increased as the cycle progressed from days 3 to 15 and then declined in early pregnancy. Further changes in the processing of phospholipids is likely to occur later in gestation. In a companion experiment conducted alongside that reported here, ewes were fed a similar LA-supplemented diet and monitored following DEX-induced labour, initiated on day 139 of pregnancy. In these ewes circulating maternal PGFM and fetal PGE_2 concentrations were higher on the high-LA diet following but not preceding the DEX infusion (Elmes *et al.* 2003). Therefore the dietary effect of LA may only become apparent when PG production is upregulated over a sufficiently long period of time for precursor availability to become rate limiting.

In conclusion, these experiments provide evidence that a diet supplemented with the *n*-6 PUFA LA can increase PG production in both maternal and fetal tissues of the ovine placenta. These findings support ideas

that a high *n*-6 diet may influence fetal and placental development, and may also play a role in determining the timing of birth.

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