Polyunsaturated fatty acids modulate prostaglandin synthesis by ovine amnion cells in vitro

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Abstract

Diets or supplements high in n-3 and n-6 polyunsaturated fatty acids (PUFAs) have been shown to influence the timing of parturition. PUFAs are substrates for prostaglandin (PG) synthesis, and PGs play central roles in parturition. Hence, the effects of altering PUFA composition may be mediated through alterations in the type and relative quantities of PGs synthesised. Therefore, we have investigated the effects of a range of n-3 and n-6 PUFAs in vitro on PG synthesis by amnion cells of late gestation ewes. The n-6 PUFA, arachidonic acid (20:4, n-6), increased synthesis of two-series PGs. Degree of stimulation induced by the n-6 PUFAs was dependent on the position of the PUFA in the PG synthetic pathway, i.e. PG production of the two-series (principally prostaglandin E2: PGE2) increased progressively with longer chain PUFAs. Effects of n-3 PUFAs on output of PGE2 were more modest and variable. The two shorter chain n-3 PUFAs, α-linolenic acid (18:3, n-3) and stearidonic acid (18:4, n-3), induced a small but significant increase in PGE2 output, while the longest chain n-3 PUFA docosahexaenoic acid (22:6, n-3) inhibited PGE2 synthesis. Dihomo-gamma-linolenic acid (20:3, n-6), the PUFA substrate for synthesis of one-series PGs, induced an increase in PGE1 generation and a decrease in PGE2 and PGE3 outputs. Hence, we have demonstrated that PUFA supplementation of ovine amnion cells in vitro affects the type and quantity of PGs synthesised.

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Introduction

The saturated fat content of the human diet has been implicated as a major risk factor in cardiovascular disease, so the Department of Health (DoH) recommends that UK consumers reduce saturated fat intake in favour of unsaturated fats (DoH 1994). Although diets high in polyunsaturated fatty acids (PUFAs) are purportedly healthier, quantities of PUFAs consumed are generally far in excess of those needed for normal physiological functioning. This is particularly so with regard to the many supplements rich in PUFAs, which are utilised by women of reproductive age to alleviate symptoms of premenstrual syndrome (Bendich 2000) and rheumatoid arthritis (Remans et al. 2004, Goldberg & Katz 2007).

PUFAs are grouped into families according to the position of the first double bond along the hydrocarbon chain, and are hence known as n-6 and n-3 PUFAs. Both n-6 and n-3 PUFAs are precursors for a range of signalling molecules including prostaglandins (PGs; Smith 1989, 1992, Lands 1992, Smith et al. 2000, Tapiero et al. 2002). Arachidonic acid (AA, 20:4, n-6) is the immediate precursor for the two-series PGs, while the three-series PGs are derived from eicosapentaenoic acid (EPA, 20:5, n-3) and the one-series PGs originate from dihomo-γ-linolenic acid (DGLA, 20:3, n-6) (Lands 1992). The amounts and relative proportions of n-6 and n-3 PUFAs in the diet may thus alter both the amount and type of PGs synthesised.

PGs influence many key processes in reproduction including ovulation (Carvalho et al. 1989), luteolysis (McCracken et al. 1970, Auletta & Flint 1988) and parturition (Thorburn 1991, Whittle et al. 2000, Challis et al. 2002, Mitchell & Taggart 2009) in numerous species (Weems et al. 2006, Jabbour et al. 2009). It is generally accepted that the onset of labour in most mammalian species including sheep is associated with an increase in n-6-derived two-series PGs (Challis et al. 2000, Olson 2003, Myatt & Sun 2010) produced by the intrauterine tissues (placenta, amnion, chorion, decidua/endometrium and myometrium) (sheep: Rice et al. 1990, 1995, Thorburn 1991, McLaren et al. 1996, 2000; guinea pig: Welsh et al. 2005; human: Slater et al. 1999, Sawdy et al. 2000). Rats, humans and sheep fed a high n-6 PUFA diet have been shown to have shortened pregnancies (Olsen & Secher 1990, Olsen et al. 1991, Olsen et al. 1991, Elmes et al. 2005), while animals (e.g. sheep) and humans (e.g. Faroese women and Greenland Eskimos) consuming diets rich in n-3 PUFAs showed delayed...
onset of uterine contractions at parturition and hence longer gestation periods (Horrobin & Manku 1990, Olsen et al. 1991, Baguma-Nibasheka et al. 1999, Szajewska et al. 2006). More recent studies in humans have also tended to support a role for dietary n-3 PUFAs in the timing of parturition with a diet devoid of fish during pregnancy being associated with a higher risk of pre-term delivery (Olsen et al. 2006), while a diet that included a modest intake of fish during gestation seemed to decrease the risk of spontaneous pre-term delivery (Olsen et al. 2007). A possible explanation for the fish diet-induced delayed onset of parturition could be that the three-series PGs generated from n-3 PUFAs have less biological potency than their two-series counterparts generated from n-6 PUFAs substrates. However, it is important to recognise that this is not a universal phenomenon as anti-aggregatory studies in rabbit platelets have shown that two-series PGs are less potent than their one- and three-series counterparts (Kobzar et al. 1993).

PGs regardless of series exert their actions via common receptors, i.e. PGE1, PGE2 and PGE3 exert their actions via the various PGE receptors, EPs 1–4 (Coleman et al. 1994, Sugimoto & Narumiya 2007, Furuyashiki & Narumiya 2009). A possible explanation for the differences in potency of action of different series PGs could lie in their relative affinities for their respective PG receptors. A report that PGE2 has greater affinity for EP receptors when compared to PGE3 in human embryonic kidney 293 cells tends to support this contention (Wada et al. 2007). On the other hand, thromboxanes A2 (TXA2) and A3 (TXA3) were reported to be equipotent in inducing aggregation despite TXA2 exhibiting a greater affinity for the cognate receptor when compared to TXA3 (Wada et al. 2007). These results illustrate how difficult it is to extrapolate and hence predict relative effects of the different series PGs in different biological situations.

Apart from acting as substrates for PG endoperoxide synthase (PTGS) enzymes (Lands 1992, Smith 1992), PUFAs also affect the expression (in bovine/ovine uterus: n-6 PUFAs: Sheldrick et al. 2007; n-3 PUFAs: Ma et al. 2000) and activities of these enzymes (Smith 2005). The increasing production of PGs by the feto-placental tissues during the 15–20 days prior to parturition is key to the successful initiation and progression of labour in the ewe (Olson et al. 1985, Thorburn 1991, Gyomorey et al. 2000). The increased production of PGs in intrauterine tissues towards the end of gestation could (at least in part) be due to increased expression/activity of PTGS1 and PTGS2 (McLaren et al. 2000, Whittle et al. 2000).

PG synthesis in the ovine amnion at term would appear to depend mainly on PTGS1 as it was found to be highly expressed in amniotic tissue from ewes in spontaneous labour, whereas PTGS2 was not detected (McLaren et al. 1996, 2000). In ovine foetal membranes, levels of PTGS1 were highest within the amnion (e.g. ~25-fold higher than in the chorion) (McLaren et al. 2000). PTGS1 was also found to be highly expressed within the guinea pig amnion, and its preferential inhibition was associated with prolonged gestation (Welsh et al. 2005). PUFAs derived from the diet and released from storage in the phospholipid component of cell membranes by the actions of phospholipase A2 (PLA2), are implicated as a major source of substrate for PTGS enzymes. This is substantiated by studies in the human, which show a striking loss of AA, 20:4, n-6 from phosphatidylethanolamine and phosphatidylinositol of amnion during early labour (Okita et al. 1982) and an increase in specific activity of PTGS in the amnion after spontaneous onset of labour (Okazaki et al. 1981). This is associated with increased metabolism of AA, 20:4, n-6 via PTGS to two-series PGs by amnion cells (Bennet et al. 1993). The observation that the ovine amnion contained greater PLA2 activity than either the myometrium or the placenta (Grieses & Liggins 1976) and that induction of labour with corticotrophin induced a marked increase in amniotic PLA2 activity (Grieses & Liggins 1976) provides further evidence to support a role for PLA2-derived AA, 20:4, n-6 metabolism to two-series PGs in inducing ovine parturition. Of the many different isoforms of PLA2, a 85 kDa cytosolic PLA2 (cPLA2) has been localised within the human amnion (Myatt & Sun 2010), and its pivotal role in parturition has been amply demonstrated by the fact that cPLA2 null mice were unable to deliver offspring (Uozumi et al. 1997).

From the preceding, it is clear that PG generation by the amnion has an effect on parturition in the ewe. However, there is a paucity of information as to how PUFAs either in vivo or in vitro influence PG output from the amnion. Hence, we have addressed this deficit by investigating the effects of n-6 or n-3 PUFAs in vitro on PG synthesis by amnion cells derived from late gestation ewes. Owing to the possible complex interactions between PUFAs, we have restricted the scope of the present study to only explore the effects of individual PUFAs in vitro on PG generation by ovine amniotic cells.

**Results**

**PG production**

In this study, effects of PUFA supplementation on PG synthesis in the ovine amnion were evaluated by measuring changes in PGE2 in cell culture medium. PGE2 was chosen as a representative parameter for perturbations of PG synthesis in the amnion, as preliminary analysis showed the amount of PGE2 synthesised to be considerably greater than that of PGF2α during the same 24 h incubation period (PGE2, 0.93 ± 0.06 ng/ml per 250 000 cells per 24 h; PGF2α, 0.083 ± 0.004 ng/ml per 250 000 cells per 24 h; ratio 11.2:1). These results were derived from multiple incubations (24 wells) of amnion cells derived from three sheep.
Effects of n-6 PUFA supplementation in vitro on PG production

The dose–response to AA, 20:4, n-6 was investigated over a comprehensive range of concentrations (0.1–1000 μM; Fig. 1). PGE2 synthesis reached maximum levels at a dose of 100 μM AA, 20:4, n-6 (184 ng/ml ± 22.6 PGE2 over 45 h of culture). Above this concentration, PGE2 synthesis declined, and the viability of the cells was compromised. Hence, a concentration of 100 μM PUFA was used for all subsequent experiments. Concentrations of up to and including 100 μM PUFA (either n-6 or n-3) had no effect on cell viability compared to untreated control cells.

Figure 2 shows that, when supplemented with a variety of n-6 PUFAs (100 μM) for 45 h, ovine amnion cells cultured in vitro increased production of two-series PGs compared to untreated control cells. Enhancement of PG production varied considerably with each different n-6 PUFA supplemented: control < ω-linolenic acid (LA, 18:2, n-6) < γ-linolenic acid (GLA, 18:3, n-6) < dihomo-γ-linolenic acid (DGLA, 20:3, n-6) < arachidonic acid (AA, 20:4, n-6). Cells treated with 20 μM PUFAs responded in a similar manner to those treated with 100 μM PUFAs, but the magnitude of response was smaller (data not shown).

Responses to in vitro supplementation with n-3 PUFAs

When cells were supplemented with PUFAs of the n-3 family, they did not respond in the same manner as the corresponding members of the n-6 PUFA family with respect to PG synthesis (Fig. 3). Shorter chain PUFAs ω-linolenic acid (ALNA), 18:3, n-3 and stearidonic acid (SA), 18:4, n-3 were stimulatory, while EPA, 20:5, n-3 was without significant effect and the longer chain PUFA docosahexaenoic acid (DHA), 22:6, n-3 was inhibitory. Compared with responses to different members of the n-6 family, the absolute differences in PGE2 synthesis were, however, small (note the differences in scale between Figs 2 and 3).

Detection of different isoforms of PGE in culture medium using specific enzyme immunoassay and HPLC

Detection of PGE1 by enzyme immunoassay

Figure 4 shows that ovine amnion cells when supplemented with a variety of n-6 PUFAs (100 μM) for 45 h in vitro increased production of one-series PGs compared to untreated control cells. Enhancement of PGE1 synthesis varied considerably with each different n-6 PUFA supplemented: control < LA, 18:2, n-6 < GLA, 18:3, n-6 < DGLA, 20:3, n-6 < AA, 20:4, n-6.

Detection of alternative isoforms of PGE after separation by HPLC

Three different isoforms of PGE in samples of cell culture medium were measured by RIA following separation of the different isoforms by HPLC.

Results from analysis of cell culture medium by HPLC demonstrate the presence of almost equal quantities of all three PG isoforms in culture medium from control cells. Cells supplemented with 100 μM AA, 20:4, n-6 produced predominantly two-series PGs, while those supplemented with 100 μM DGLA, 20:3, n-6 produced predominantly one-series PGs and those supplemented with 100 μM EPA, 20:5, n-3 produced significantly greater quantities of three-series PGs as shown in Table 1.

Discussion

The ovine amnion is an important site for PG synthesis in both pre-term and term labour (Olson et al. 1986, McLaren et al. 1996, 2000, Palliser et al. 2006). Hence, the present study was designed to investigate whether
PUFAs in vitro could affect PG synthesis by isolated ovine amnion cells in primary culture. Many previous studies have involved the use of an immortalised amnion cell line (WISH cells – reviewed in Pavan et al. (2003)), but there is a paucity of data derived from experiments using primary cultures of amnion cells. PGE2 was chosen as a representative parameter for perturbations of PG synthesis in the amnion, as preliminary analyses showed PGE2:PGF2α synthesis to be >10:1. Okazaki et al. (1981) similarly reported that the ratio of PGE2:PGF2α in human amnion tissues after spontaneous labour and at caesarean section before the onset of labour was in the region of 30:1. We show here that the addition of different PUFAs in vitro had major effects on the levels of PGE2 production in vitro. These results suggest that PUFAs (dietary and supplements) could potentially alter the timing of parturition through changes in PG synthesis.

The present study demonstrated that, when supplemented with selected n-6 PUFAs for 45 h, amnion cells from late gestation ewes, cultured in vitro, increased synthesis of two-series PGs compared to untreated control cells. All the n-6 PUFAs, except LA, 18:2, n-6, increased PGE2 synthesis. Significantly, the position of the supplemented PUFAs in the PG synthetic pathway governed the extent to which PGE2 synthesis was enhanced (Fig. 2), with PGE2 production increasing progressively with the more elongated PUFAs. In agreement with the present studies, Arntzen et al. (1998) obtained similar responses in human decidual cell cultures supplemented with 50 µM n-6 PUFAs, i.e. LA, 18:2, n-6 had no significant effect on PGE2 synthesis, whereas all other n-6 PUFAs stimulated PGE2 (and PGF2α) synthesis in the order: GLA, 18:3, n-6 < DGLA, 20:3, n-6 < AA, 20:4, n-6.

It is clear that elongation and desaturation of n-6 PUFAs to AA, 20:4, n-6 will be rate limited by the presence/absence of relevant enzymes (Δ-5 desaturase, elongase and Δ-6 desaturase) within the ovine amnion. Thus, supplementation with LA, 18:2, n-6 or GLA, 18:3, n-6 may lead to a build-up of DGLA, 20:3, n-6. As DGLA, 20:3, n-6 is the precursor for the one-series PGs (as well as being an intermediate in the two-series synthetic pathway), this could potentially lead to an increase in the production of one-series PGs (Belch & Hill 2000). Alternatively, DGLA, 20:3, n-6 may itself be metabolised to various hydroxy derivatives, which could feed back and inhibit the desaturation and elongation enzymes in the synthetic pathway. Although there is little information regarding the expression of these enzymes in ovine amnion tissue, our results suggest that they are likely to be present and active, as we observed an increase in PGE (PGE1 and PGE2, Figs 2 and 4) synthesis following supplementation with GLA, 18:3, n-6 and DGLA, 20:3, n-6. This notion is indirectly supported by the observed increase in Δ-6 desaturase activity in the ovine placenta during the latter stages of gestation (Shand & Noble 1981).

When effects of n-3 PUFAs on two-series PG synthesis were compared to effects of equivalent n-6 PUFAs, there were very clear differences in responses of amnion cells. There was a small increase in synthesis of PGE2 with the two shorter chain n-3 PUFAs ALNA, 18:3, n-3 and SA, 18:4, n-3, and a decrease in PGE2 synthesis by the longest chain n-3 PUFAs DHA, 22:6, n-3 (Fig. 3). EPA, 20:5, n-3 on the other hand had no effect on PGE2 synthesis.
Table 1 Effects of n-6 and n-3 polyunsaturated fatty acids (PUFAs) in vitro on synthesis of different isoforms of prostaglandin E (PGE) in ovine amnion cells. Cell culture medium (DMEM/F-12 with 0.1125% fatty acid-free BSA and 0.1 ml/100 ml ITS media supplement) was supplemented with treatments: 0 (CONT) or 100 μM PUFAs (dihomo-γ-linolenic acid (DGLA, 20:3, n-6), arachidonic acid (AA, 20:4, n-6) or eicosapentaenoic acid (EPA, 20:5, n-3)) for 45 h. Results show PGE measured by RIA after concentration of individual peaks separated by HPLC. In vitro treatments were replicated in four wells of cells in each experiment. Each experiment was repeated three times with cells isolated from an individual sheep on each occasion. PG synthesis is expressed as (mean) percentage PGE/45 h. For HPLC separation, the incubation medium from all four wells that received the same treatment was pooled. Separated fractions containing different isoforms were freeze-dried before being subjected to RIA for PGE. This yielded a single value for each PGE isoform for each experimental animal.

<table>
<thead>
<tr>
<th>PUFA</th>
<th>Mean percentage of each isoform of PGE in samples</th>
<th>Total ng PGE/ml per 45 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PGE₁</td>
<td>PGE₂</td>
</tr>
<tr>
<td>CONT</td>
<td>36.0±5.0</td>
<td>30.7±7.5</td>
</tr>
<tr>
<td>DGLA, 20:3, n-6</td>
<td>76.0±4.0*</td>
<td>8.7±1.4</td>
</tr>
<tr>
<td>AA, 20:4, n-6</td>
<td>20.7±3.2</td>
<td>64.3±6.6*</td>
</tr>
<tr>
<td>EPA, 20:5, n-3</td>
<td>29.7±2.2</td>
<td>28.7±1.9</td>
</tr>
</tbody>
</table>

Significant differences between control and treatments are indicated by *P<0.01 (statistical analyses carried out on numerical data).

significantly enhanced PGE₁ generation (Table 1). However, a simple shift from synthesis of two- to three-series PGs is just one aspect of a complex situation. Previous studies have shown that supplementation with n-3 PUFAs may actually inhibit two-series PG production (Herrera 2002, Bagga et al. 2003). Simple, competitive inhibition of key elongase and desaturase enzymes in the PUFA elongation pathway for PUFAs derived from the diet is the most obvious explanation. However, Burdge & Wootton (2002) and Burdge et al. (2002) reported limited ability of humans to elongate and desaturate ALNA, 18:3, n-3, indicating that the type of n-3 PUFAs supplemented in the diet, and hence available to the cells, becomes very significant. There would also be competition between n-3 and n-6 PUFA intake for incorporation into phospholipids within cell membranes. For instance, in rat erythrocytes, increased dietary intake of n-3 PUFAs (fish oil supplement) led to significant changes in membrane composition, which resulted in increased amounts of AA, 20:4, n-6 being stored in membrane phospholipids, which could therefore potentially result in decreased synthesis of two-series PGs (Escudero et al. 1998). Furthermore, during PG synthesis, competition for PTGS enzymes could result in a decrease in two-series PGs with increased n-3 substrate (EPA, 20:5, n-3) availability (Allen & Harris 2001, Wada et al. 2007). Since EPA, 20:5, n-3 is both a poor substrate for PTGS1 (Smith 2005) and an inhibitor of enzyme activity (Lands 1992), it is unlikely that the three-series PGs are generated as a result of PTGS1 activity. In terms of PTGS2, EPA, 20:5, n-3 is a poor inhibitor of its activity (Wada et al. 2007). Taken together, it would seem more likely that the n-3 PUFA-induced decrease in PGE₂ observed in this study (Fig. 4) was due to decreased PTGS1 rather than PTGS2 activity.

Increasing inhibitory effects of n-3 PUFAs with increasing chain length have also been reported by other workers (Hwang et al. 1988), with EPA, 20:5, n-3 having a greater inhibitory effect on PG synthesis than ALNA, 18:3, n-3. More recently, similar observations have been reported in human decidual cell cultures where DHA, 22:6, n-3 was found to be more potent than EPA, 20:5, n-3 in inhibiting two-series PG synthesis (PGF₂α and PGE₂) stimulated by interleukin-1β (Roman et al. 2006). In addition, in the same study, Roman et al. (2006) reported that one of the rate-limiting enzymes associated with the synthesis of PGE₂, namely microsomal PG synthase 1, was also inhibited by DHA, 22:6, n-3. While Roman et al. (2006) reported PGE₂ decreases with both EPA, 20:5, n-3 and DHA, 22:6, n-3, we have only observed significant inhibitory effects of n-3 PUFAs, with DHA, 22:6, n-3. Species and tissue differences probably account for the observed variations in the two-series PGs response between the studies. Another possibility that needs considering is that the concentrations of PGs measured with the PGE₂ RIA may actually be a combined value, representing a decrease in two-series PGs and an increase in three-series PGs. Most studies to date make no attempt to quantify changes in one- and three-series PGs following supplementation with PUFAs. This is a major omission, which we have gone some way towards addressing in the present study where we show that n-6 PUFAs do indeed induce increases in two-series PGs (Figs 1 and 2 and Table 1). Conversely, n-3 PUFAs increase three-series PGs, while at the same time, they decrease two-series PGs (Table 1 and Fig. 4). Additional support for the idea that the type and quantity of PGs produced depend upon the type of PUFAs used for supplementation comes from our observations that DGLA, 20:3, n-6 leads to an apparent increase in PGE₁ output while decreasing both PGE₂ and PGE₃ outputs from ovine amnion cells (Table 1).

In summary, we have demonstrated that supplementation in vitro with n-6 PUFAs elicited profound stimulatory effects on PG synthesis in ovine amnion cells. A long-chain n-3 PUFA, DHA, 22:6, n-3, was inhibitory in terms of PGE₂ production, whereas shorter chain n-3 PUFAs produced a modest stimulatory effect on PGE₂ output. The complexity of the processes involved between dietary intake and tissue specific modulation of PG synthesis makes it very difficult to reach simple conclusions as to the likely effects of changes in dietary PUFA composition on functions in specific cells (Galli et al. 1993). The mechanisms underlying either the increased gestational length or the reduced risk of spontaneous pre-term delivery induced by the high n-3 PUFA diet are poorly understood. However, increasing n-3 PUFA supplementation and decreasing n-6 PUFA intake may be a way forward to reduce the risk of pre-term birth.
understood (Wathes et al. 2007). One possible explanation for these effects could be that the n-3 PUFAs used were purchased from Sigma–Aldrich. The n-6 PUFAs used were LA (18:2, n-6), γ-linolenic acid (GLA, 18:3, n-6), DGLA (20:3, n-6) and AA (20:4, n-6). The n-3 PUFAs were ALNA (18:3, n-3), SA (18:4, n-3), EPA (20:5, n-3) and DHA (22:6, n-3). The fatty acids were initially dissolved in organic solvent (100 mM in ethanol) and then further diluted in DMEM/F-12 containing 0.1125% (w/v) fatty acid-free BSA (to provide a carrier) and 0.1% (v/v) ITS media supplement (containing 0.5 mg/ml insulin, 0.5 mg/ml transferrin and 0.5 μg/ml selenium). All PUFAs were supplemented in the concentration range of 0.1–1000 μM, where the final concentration of the organic solvent was <0.001%. DMEM/F-12 containing 0.1125% fatty acid-free BSA and 0.1% ITS was used as the diluent for all treatments in order to standardise the fatty acid composition of the cell culture medium.

Viability of cells following treatment with PUFAs for 45 h was determined by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) from Promega (Promega Corporation) according to the manufacturer’s instructions (Si et al. 2005).

**Experimental protocol**

Cells isolated from amnion tissue were cultured to confluence over a period of 6 days (where day 0 = start of culture period) in DMEM/F-12 containing 10% FCS at a temperature of 39 °C in an atmosphere of 5% (v/v) CO₂. Medium was changed every 48–72 h. At the start of day 6, culture medium was replaced with DMEM/F-12, supplemented with 0.1125% fatty acid-free BSA and 0.1 ml/100 ml ITS media supplement, for a period of 3 h to remove any fatty acids present in the FCS (Cheng et al. 2004). Cells were then cultured in the presence of 0, 20 or 100 μM n-6 PUFAs (LA, 18:2, n-6; GLA, 18:3, n-6; DGLA, 20:3, n-6 or AA, 20:4, n-6) or 20 or 100 μM n-3 PUFAs (ALNA, 18:3, n-3; SA, 18:4, n-3; EPA, 20:5, n-3 and DHA, 22:6, n-3) for 45 h, as this time point was shown to be optimal for assessing PG generation following PUFAs supplementation in the ovine amnion (Cheng et al. 2003). Four wells of cells were subjected to each different in vitro treatment. This protocol was repeated at least three times using amnion cells from an individual ewe on each occasion. Spent culture medium was stored at −20 °C until analysis of PGs by RIA, enzyme immunoassay (EIA) and/or HPLC.

**PG RIA**

PGE₂ and PGF₂ₐ in the spent medium were quantified by RIA as validated and described previously for samples of ovine origin (Cheng et al. 2003). The tritiated tracers of PGE₂ ([5, 6, 8, 11, 12, 14, 15 (n)-³H] PGE₂) and PGF₂ₐ ([5, 6, 8, 11, 12, 14, 15 (n)-³H] PGF₂ₐ) were obtained from Amersham International, and the PG standards were obtained from Sigma–Aldrich. The PG antisera (anti-PGE₂ and anti-PGF₂ₐ) were a kind gift from Prof. N L Poyser (University of Edinburgh, Edinburgh, UK; Poyser 1987). Respective cross reactivities of PGs have been reported previously (Cheng et al. 2005a, 2005b) and were estimated to be as follows in the present study: anti-PGE₂, 23% with PGE₁, 100% with PGE₂ and 15% with PGE₃; anti-PGF₂ₐ, 25% with PGF₁ₐ, 100% with PGF₂ₐ and 18% with PGF₃ₐ.
The RIAs for PGE 1 and PGE 3 were carried out using the anti-PGE2 antiserum and the 3H-PGE2 tracer but with authentic PGE1 and PGE3 standards (Fig. 5) as described for the assay of PGF1α and PGF 3α (Cheng et al. 2005a). The samples were diluted appropriately in 0.05 M Tris buffer containing 0.1% (w/v) gelatin and 0.01% (w/v) sodium azide and assayed as previously described (Cheng et al. 2003). The limit of detection was 1 pg/tube for PGF2α, 2 pg/tube for PGE2 and 100 pg/tube for PGE 1 and PGE 3. The cross reactivity of anti-PGE 2 with PGF2α and anti-PGF2α with PGE2 was 0.8%.

HPLC
PGE isomers were separated using HPLC methodology as described previously for the separation of PGF isomers (Cheng et al. 2005a) where the system details were as follows: a Beckman C18 Ultrasphere 5 μm ODS2 column (4.6 × 25 cm) with a compatible C18 guard column and a solvent system of 17 mM phosphoric acid:acetonitrile (74:26 v/v) was used in conjunction with a Beckman Gold HPLC system (Brea, CA 92622-9855, USA) equipped with a UV detector (Beckman Gold 166), a fraction collector (Beckman SC100), a gradient pump (Beckman 125 pump) and a Beckman Gold Nouveau data acquisition system (V1.1.). In order to determine the retention time of PGE isomers, PGE1, PGE2 and PGE3 standards (20 μl of 50 μg/ml) in acetonitrile were injected onto the column, where the flow rate was 1 ml/min and the wavelength of detection was 196 nm. In this system, the average retention times of the three PGE isomers were as follows: PGE3, 12.5 min; PGE2, 19 min; and PGE1, 22 min.

Samples of cell culture medium from cultures that had been supplemented with the same in vitro treatments were pooled, and various isoforms were separated by HPLC (Cheng et al. 2005a). The three isoforms were identified by comparison to commercial standards of PGE1, PGE2 and PGE3. Fractions containing individual peaks were then pooled and freeze-dried, before being reconstituted in RIA buffer. Concentrations of the relevant PGE isoforms were derived from standard curves plotted for individual PGE isoforms (Fig. 5).

Enzyme immunoassay
PGE1 EIA assay kits were obtained from Assay Designs, Inc., Ann Arbor, MI, USA. Cross reactivities of the alternative isoforms of PGE with these assay kits were 100% for PGE1, 6.5% for PGE2 and 2.22% for PGE3. This was confirmed by testing a range of concentrations of PGE2 and PGE3 alongside the standards of PGE1 in pre-assays.

PGE1 levels were estimated in a number of samples of spent cell culture medium. Samples were diluted appropriately prior to assay according to the manufacturer’s instructions where the threshold for detection was 5.5 pg/ml PGE1.

Statistical analysis
Results are quoted as the mean ± s.e.m. (ng/ml per 45 h) of PGE2 concentrations in culture medium unless otherwise stated. The n value quoted refers to the number of sheep and equals three unless otherwise stated. In vitro treatments were replicated in four wells of cells in each experiment. Each experiment was repeated at least three times with cells isolated from an individual sheep on each occasion. PG data were analysed by a general linear model ANOVA programme using a repeated-measures design (SAS Inc., Cary, NC, USA) unless otherwise stated. The Bonferroni test was used to make post hoc comparisons. A significance (P) value of <0.05 was taken to indicate a significant difference between treatments.

Declaration of interest
The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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