Stimulation of DNA synthesis in cultures of ovine mammary epithelial cells by insulin and insulin-like growth factors

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ABSTRACT

Ovine mammary epithelial cell clumps (30–90 μm) were plated onto attached gels of rat tail collagen in serum-free medium. Synthesis of DNA by these cultures could be stimulated by insulin-like growth factor-I (IGF-I) with a median effective dose of 5 μg/l, irrespective of stage of pregnancy. The time-course of response, however, was significantly slower in cells prepared from mammary tissue of non-pregnant and early pregnant sheep compared with sheep later in pregnancy. IGF-II had approximately 10% of the potency of IGF-I in stimulating DNA synthesis. Insulin acted over a wide concentration range and produced a maximum rate of stimulation not significantly different from that produced by IGF-I. These results are consistent with actions through the type-I IGF receptor although insulin may also act through its own receptor, possibly stimulating local IGF-I production. It is concluded that IGF-I is an important mitogen for ovine mammary epithelial cells.


INTRODUCTION

Recent evidence suggests that the pharmacological concentrations of insulin necessary for the maintenance and stimulation of cell growth in many tissues in vitro could have their effects via the type-I insulin-like growth factor (IGF-I) receptor (Flier, Usher & Moses, 1986). Maintenance of mammary tissue in vitro, using methods of whole organ (Prop, 1961), explant (Rivera & Bern, 1961) and cell culture (Imagawa, Tomooka & Nandi, 1982) has required non-physiological concentrations of insulin. Stockdale, Juergens & Topper (1966) showed that insulin (5 mg/l; 0-83 μmol/l) was a necessary and sufficient stimulus for DNA synthesis in explants of mouse mammary gland. The use of cell culture systems now indicates a role of IGF-I, acting at physiological concentrations, in stimulating the growth of epithelial cells derived from human breast cancer (Furlanetto & DiCarlo, 1984; Myal, Shiu, Bhaumick & Bala, 1984), normal and tumorous mouse mammary glands (Imagawa et al. 1982) and normal bovine mammary glands (Baumrucker, 1986; Shamay, Cohen, Niwa & Gertler, 1988).

Ruminant mammary gland contains binding sites for insulin (Campbell, Frey & Baumrucker, 1987) and also type-I and type-II IGF receptors, preferentially binding IGF-I and IGF-II respectively (Dehoff, Elgin, Collier & Clemmons, 1988; Disenhaus, Belair & Djiane, 1988). We have therefore compared the effects of IGF-I, IGF-II and insulin on DNA synthesis by mammary cells from sheep which were in various physiological states, using a serum-free system. Preliminary results were reported by Winder & Forsyth (1986).

MATERIALS AND METHODS

Materials

Twenty-four-well culture dishes were from Sterilin, Feltham, Middx, U.K. Medium 199 (M199), with Earle’s modified salts, penicillin G (0-2 × 10^6 units/l), streptomycin sulphate (100 μg/l), phenol red (20 mg/l) and sodium bicarbonate (1·1 g/l), was from Gibco, Paisley, Strathclyde, U.K. Bovine serum albumin (BSA; fraction V, 98–99% albumin), bovine insulin (26·8 U/mg), 3,5-diaminobenzoic acid dihydrochloride (DABA) and multiplication-stimulating activity (rat IGF-II) were from Sigma, Poole, Dorset, U.K. BSA, prepared from pasteurized serum by charcoal treatment and dialysis to reduce low molecular weight substances, contained no fatty acid. By radioimmuno-
assay it was shown to contain less than 1 ng IGF-I/ mg. [6-3H]Thymidine (1 mCi/mmol) and human recombinant IGF-I were from Amersham International plc, Amersham, Bucks, U.K. Sodium acetate, calf thymus DNA and Hepes were from BDH, Poole, Dorset, U.K. Ovine prolactin (NIH-P-S-12; 35 IU/mg) and bovine growth hormone (NIH-P-B-15; 0.69 IU/ mg) were gifts from NIADDK, Bethesda, MD, U.S.A. Mouse epidermal growth factor was a generous gift from Dr K. D. Brown (AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, U.K.). Ovine placental lactogen was purified by the method of Thordarson (1984) (see Mellor, Flint, Vernon & Forsyth, 1987). Highly purified human IGF-II was from Sera-lab, Crawley Down, Sussex, U.K.

Cell culture

Poll–Dorset ewes provided most of the mammary tissue and were non-pregnant or primigravid; some Derbyshire Gritstone and Suffolk-cross ewes were also used. Sheep were killed by stunning with a captive bolt pistol followed by exsanguination, or by using an overdose of sodium pentobarbitone. Udder tissue was removed immediately and kept at room temperature until processed, within 10 to 30 min. Mammary epithelial cells were prepared by collagenase–hyaluronidase digestion as described by Mackenzie, Forsyth, Brooker & Turvey (1982), with the modification that filtration through nylon gauze (Henry Simon, Stockport, Cheshire, U.K.) was used to obtain cell clumps in the 30–90 μm size range, free of single cells. All serum-containing media were replaced with a serum-free medium prepared by adding 20 mmol Hepes/l, 5 mmol sodium acetate/l and 5 g BSA/l to M199. A preliminary experiment using tissue from three sheep showed this concentration of BSA to be optimal for DNA synthesis in medium without hormones and growth factors. Cell clumps were plated onto attached gels of 0.5% (w/v) rat tail collagen (Bornstein, 1958) in well culture dishes (24 × 16 mm diameter) in 0.4 ml serum-free attachment medium. This contained ovine fibronectin (10 mg/l) purified from sheep plasma by the method of Ruoslahti, Vuento & Engvall (1978) using an Affi-gel gelatin affinity column (Bio-Rad, Watford, Herts, U.K.). After 18 h, attachment medium was replaced with test media containing hormones and growth factors, and the media were changed every 24 h. This was found to be necessary for sustained DNA synthesis. Culture was in air at 37 °C. The epithelial origin of cultured cells was demonstrated by showing that all cells reacted with a mouse monoclonal antibody to cytokeratin, supplied by Amersham International plc (code RNP 1100).

Incorporation of thymidine into DNA

DNA synthesis was estimated by the incorporation of [3H]thymidine (1 μCi/well) over successive 24-h periods. After 24 h in the presence of [3H]thymidine the gels, with cells attached, were removed, washed briefly in ice-cold NaCl (0.9%, w/v) and frozen at −20 °C until assayed. Thawed gels were dissolved by adding 1 ml acetic acid (2 mol/l) and incubating for 30 min at 37 °C. The resulting cell suspension was centrifuged at 1820 g for 30 min at room temperature, the supernatant was removed and the pellet resuspended in 1 ml ice-cold distilled water. With the samples at 4 °C, 200 μl perchloric acid (2 mol/l) was added, mixed and allowed to stand for 20 min. The tubes were centrifuged (1820 g) at 4 °C and the supernatant was removed. The DNA-enriched pellets were assayed for DNA by the method of Hinegardner (1971) using 4% (w/v) DABA. Fluorescence was measured in a Perkin Elmer LS 2B fluorimeter. After fluorescence estimation, 1 ml of each sample was counted in a Packard Instrument Tri-Carb B2450 beta counter in 10 ml Instagel (Packard, Pangbourne, Berks, U.K.) against external standards. The means of triplicate observations for each treatment were calculated and expressed as d.p.m./μg DNA. Incorporation of [3H]thymidine was linear up to 24 h.

Autoradiography

Gels with cells attached were fixed in 5% (v/v) formaldehyde in 95% (v/v) alcohol, washed in 70% alcohol, taken through graded alcohols to distilled water, dried onto subbed slides and processed as described by Rogers (1967). Slide-mounted gels were dipped in emulsion (Ilford Nuclear Research; emulsion K2 diluted 1:1 in 2% (v/v) glycerol in distilled water) at 43 °C, drained briefly, placed on an ice-cold metal block to gel and allowed to dry. The autoradiographs were exposed for 48 h at −20 °C, developed with a 1:1 (v/v) dilution of 2,4-diaminophenol dihydrochloride (BDH) in distilled water, fixed in sodium thiosulphate (30%, w/v), washed in water and counterstained with haematoxylin before being dehydrated in alcohol, cleared in xylene and mounted in DPX mountant (BDH). Labelling index was determined by counting the number of labelled nuclei from at least 400 cells per field and three fields per gel.

Presentation of results and statistical analysis

Individual sheep showed marked variation in the absolute magnitude of thymidine incorporation into DNA expressed as d.p.m./μg DNA, which could not be correlated with age, stage of pregnancy, parity, breed or season. Therefore, to enable observations from several individuals to be combined, results have
been normalized by expressing them as a percentage of the maximum response in each sheep.

Treatment differences were analysed using Student's paired or unpaired t-test or analysis of variance. Maximum Likelihood Programme (Ross, 1987) was used for analysis of dose-response curves and of time-course curves. Curves were in the form

\[ y = A + B \exp (-kx) \]

where \( y \) is per cent maximum response, \( x \) is dose or time and \( A \), \( B \) and \( k \) are constants.

RESULTS

Effects of single hormones or growth factors on DNA synthesis in sheep mammary epithelial cells

The effects of various concentrations of growth hormone (GH), prolactin, placental lactogen, insulin and IGF-I on DNA synthesis by mammary epithelial cells from a non-pregnant ewe after 5 days in culture are shown in Fig. 1. Only insulin and IGF-I stimulated any marked increase in DNA synthesis (\( P < 0.05 \) at all doses, compared with no hormone). In another experiment, epidermal growth factor (EGF) (1, 10 and 100 µg/l) produced about a twofold increase in DNA synthesis compared with a sixfold increase in response to insulin (5 mg/l; results not shown).

Time-course of response to insulin and IGF-I

Representative time-courses of response of mammary cells from non-pregnant, early pregnant and late pregnant ewes are shown in Fig. 2. The time-course of response to insulin (1 or 5 mg/l) and to IGF-I (100 or 200 µg/l) were similar. The response was more rapid in late pregnancy, reaching a maximum on days 2–3 of culture (Fig. 2c). In the non-pregnant and early pregnant ewes (Fig. 2a and b), a first significant (\( P < 0.01 \)) response compared with no hormones was seen on day 3 but the response continued to increase to days 4–5. This was examined further in Poll–Dorset sheep at days 40 (\( n = 4 \)), 75 (\( n = 4 \)) and 110 (\( n = 3 \)) of pregnancy and at term (\( n = 3 \)), compared with non-pregnant sheep (\( n = 4 \)). The response to a sub-maximal dose of IGF-I (10 µg/l) was assessed. Curves were fitted to the data from individual sheep. This
showed a significant \((P=0.003)\) difference in the constant \(k\) for tissue from non-pregnant and 40-day pregnant ewes as compared with the other stages. The time-course of response for mammary tissues from sheep after 75 days of pregnancy compared with non-pregnant or early pregnant sheep is shown in Fig. 3.

**Effect of stage of pregnancy on dose–response of DNA synthesis to IGF-I**

Using a 5-day culture period, the response of tissue from the same sheep to IGF-I at 0.01, 0.1, 1, 10 and 100 \(\mu\)g/l was compared. While the stage of pregnancy affected the time-course of DNA synthesis in response to IGF-I, the dose–response relationship was not significantly affected.

**Comparison of responses to insulin, IGF-I and IGF-II**

In initial experiments, it appeared that IGF-I was much more potent than insulin in stimulating DNA synthesis in ovine mammary epithelial cells. When lower doses of insulin were examined, however, even concentrations as low as 10 ng/l were able to stimulate DNA synthesis. The combined dose–response curves for 28 sheep are shown in Fig 4. Compared with the classic sigmoid dose–response relationship for IGF-I with a median effective dose \((ED_{50})\) of 5 \(\mu\)g/l, the response to insulin was more variable and extended over a very wide millionfold concentration range.

In tests using tissue from non-pregnant and pregnant sheep, partially purified rat IGF-II produced no dose-related stimulation of DNA synthesis by ovine mammary epithelial cells (Fig. 4). Highly purified human IGF-II was also tested in cells from a non-pregnant sheep (Table 1). It produced a significant \((P<0.05)\) stimulation of DNA synthesis at the highest dose tested showing about 10% of the potency of IGF-I.

**Labelling index and growth curves**

In order to validate the use of incorporation of thymidine into DNA as a measure of DNA synthesis, two experiments were carried out in which labelling indices were determined in sheep mammary cells. Labelling index and incorporation of thymidine into DNA were compared directly (Fig. 5a). Both methods produced essentially similar results when expressed as percentages of the maximum response obtained. In a second experiment, labelling indices were determined at 2-h intervals after continuous labelling with \([3H]thymidine\) for up to 48 h, starting from time 0, 24 and 48 h in culture (Fig. 5b). A lag of about 30 h occurred before nuclear labelling was observed (curves 1 and 2). After 4 days in culture (48 h in the presence of \([3H]thymidine\), 70% of all cells showed nuclear labelling. There was general agreement between the three curves in the extent of labelling at various time-points from the start of culture.

**DISCUSSION**

These results show that ovine mammary epithelial cells, prepared and cultured in a serum-free medium, and grown on an attached rat-tail collagen substrate, respond to human IGF-I by a marked increase in
DNA synthesis. The response was maximal at 100 μg/l with an ED_{50} of 5 μg/l and this dose–response relationship was independent of stage of pregnancy. Concentrations of total IGF-I of this order are reported in sheep plasma using acid–ethanol extraction (Pell, Johnsson, Pullar et al. 1989) and acid gel filtration (Mesiano, Young, Browne & Thorburn, 1988). More limited results suggest that human IGF-II is a less potent stimulator of DNA synthesis. The role of the abundant type-II receptors in ruminant mammary tissue (Dehoff et al. 1988; Disenhaus et al. 1988) remains therefore uncertain. While the amino acid sequences of human and bovine IGF-I are identical, human and bovine IGF-II differ at residues 32, 35 and 36 (Francis, Upton, Ballard et al. 1988).

The time-course of response to IGF-I was affected by stage of pregnancy, with a longer lag phase in tissue from non-pregnant and early pregnant sheep. Skarda, Urbanova & Bilek (1978) showed a similar pattern of response using insulin (5 mg/l) to stimulate DNA synthesis in goat mammary explants. Tissue taken from goats after 8 weeks of pregnancy showed maximum DNA synthesis in response to insulin after 2 days of culture, compared with 4 or 5 days in early pregnancy. The response of mouse mammary explants to insulin is also affected by stage of pregnancy (Oka, Perry & Topper, 1974). It is probable that different time-courses reflect the number of cells in S phase at the start of culture. Continuous labelling of cells from a 40-day pregnant ewe showed a lag of 30 h before nuclei were labelled (Fig. 5b).

These findings are consistent with the actions of IGF-I, insulin, and perhaps also IGF-II, via a single receptor, the type-I IGF receptor. Studies on human breast cancer cell lines have shown that IGF-I is mitogenic at doses 10- to 100-fold less than insulin (Furlanetto & Di Carlo, 1984) and that IGF-I acts through the type-I IGF receptor (Rohlik, Adams, Kull & Jacobs, 1987).

In the present study, insulin stimulated DNA synthesis at high and unphysiological concentrations (maximally effective at 1 mg/l). However, insulin also produced significant stimulation at concentrations too low for effective cross-reaction with the type-I
TABLE 1. Effect of human IGF-I, human IGF-II and rat IGF-II on DNA synthesis by mammary epithelial cells from a non-pregnant sheep on day 4 of culture. Values are means ± s.e.m.; n = 3

<table>
<thead>
<tr>
<th>Hormone dose (µg/l)</th>
<th>[3H]Thymidine incorporation into DNA (d.p.m./µg DNA)</th>
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</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3606 ± 336</td>
</tr>
<tr>
<td>0·1</td>
<td>5305 ± 56</td>
</tr>
<tr>
<td>1·0</td>
<td>5684 ± 284</td>
</tr>
<tr>
<td>10</td>
<td>6084 ± 281</td>
</tr>
<tr>
<td>100</td>
<td>9884 ± 232</td>
</tr>
<tr>
<td>IGF-II (human)</td>
<td></td>
</tr>
<tr>
<td>0·1</td>
<td>5037 ± 689</td>
</tr>
<tr>
<td>1·0</td>
<td>4116 ± 619</td>
</tr>
<tr>
<td>10</td>
<td>5166 ± 317</td>
</tr>
<tr>
<td>100</td>
<td>6836 ± 759*</td>
</tr>
<tr>
<td>IGF-II (rat)</td>
<td></td>
</tr>
<tr>
<td>0·1</td>
<td>4456 ± 110</td>
</tr>
<tr>
<td>1·0</td>
<td>4331 ± 273</td>
</tr>
<tr>
<td>10</td>
<td>4847 ± 77</td>
</tr>
<tr>
<td>100</td>
<td>4496 ± 158</td>
</tr>
</tbody>
</table>

*P < 0·05 compared with IGF-I and rat IGF-II at the same dose (Student’s t-test).

receptor, since we (Winder & Forsyth, 1987) and Disenhaus et al. (1988) have shown that insulin has a weak affinity for the type-I receptor in sheep mammary tissue. This suggests that insulin can act to stimulate DNA synthesis, either directly or indirectly, via its own receptor. Campbell et al. (1987) have shown changes in numbers of high affinity insulin receptors with physiological state in the mammary gland of dairy cows, maximum specific binding being observed from mid to late pregnancy.

If IGF-I is a physiological mitogen for ruminant mammary cells, it could act either as an endocrine or a paracrine/autocrine factor. The liver is a major source of circulating IGF-I and GH is a dominant factor in IGF-I control (Gluckman, Brier & Davis, 1987). In vivo studies in ruminants implicate GH in stimulating mammary growth (Cowle, Tindal & Yokoyama, 1966) and the systemic injection of bovine GH increases total circulating concentrations of IGF-I in sheep (Pell et al. 1989) and in cows (Davis, Gluckman, Hart & Henderson, 1987). Since no GH receptors have so far been demonstrated in ruminant mammary tissue (Akers, 1985), stimulation of mammary growth by GH could then be mediated indirectly through increased IGF-I production from the liver. In the human breast cancer cell line MCF-7, however, both insulin and oestradiol stimulate production of IGF-I related material (Huff, Knabbe, Lindsey et al. 1988). Using a polyclonal antibody to IGF-I we have been able to show inhibition of both basal and insulin-stimulated DNA synthesis in mammary explants from sheep (Wheatley, Morrell & Forsyth, 1988). This suggests a role for locally produced IGF-I in mammary growth, supported by the tendency for DNA synthesis to increase over time even in the absence of hormones and growth factors (Fig. 2).

Studies in the mouse and the cow have also implicated IGF-I as a major mitogen for mammary epithelial tissue. In the mouse, using cell number as an endpoint, Imagawa, Spencer, Larson & Nandi (1986) have found that EGF (or progesterone and prolactin), as well as IGF-I is required for a response to occur. In the cow (Shamay et al. 1988), IGF-I and EGF showed

![FIGURE 5](image)
synergistic effects but only in the presence of 10% fetal calf serum. These results suggest that proliferation of mammary epithelial tissue may require the synergistic actions of both systemic and local hormones and growth factors, including IGF-I.

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