Chronic control of the β - and α_2 -adrenergic systems of sheep adipose tissue by growth hormone and insulin

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1. Sheep adipose tissue retained responsiveness to catecholamines when maintained in tissue culture for 48 h; both the rate of basal lipolysis and sensitivity to β -agonists were increased after tissue culture. 2. Tissue culture in the presence of growth hormone resulted in an increased maximum response and sensitivity to the β -agonist isoprenaline, but had no effect on basal lipolysis. 3. Tissue culture in the presence of insulin increased the basal rate of lipolysis and increased the ratio of the rate of noradrenaline-stimulated/isoprenaline-stimulated lipolysis, indicating a decrease in the α_2 -adrenergic effect of noradrenaline. 4. Tissue culture in the presence of growth hormone increased ligand binding to β -adrenergic receptors. 5. Tissue culture in the absence of exogenous hormones increased ligand binding to α_2 -adrenergic receptors; this was prevented by actinomycin D and partly prevented by insulin. 6. These studies show that both growth hormone and insulin chronically modulate the adrenergic system of sheep adipose tissue; the effects of growth hormone are primarily on the β -adrenergic system, whereas insulin modulates the α_2 -adrenergic system.

INTRODUCTION

The cycle of pregnancy, lactation and weaning has a profound effect on lipid metabolism in adipose tissue; lipid reserves are usually accumulated during much of pregnancy, lost during lactation and then replenished on litter removal in rats or during the declining phase of lactation in ruminants (Bauman & Elliot, 1983; Vernon & Flint, 1984). These changes are accompanied by adaptations in the capacity of adipocytes to synthesize lipid (Vernon & Flint, 1983). In addition, there are changes in the responsiveness of adipocytes to agents which control lipolysis. In rats, lactation itself has no apparent effect on the response of adipocytes to catecholamines, but replenishment of lipid reserves after litter removal is associated with a diminished ability of catecholamines to stimulate lipolysis (Vernon & Finley, 1986). In contrast, in sheep both the response and sensitivity of adipocytes to catecholamines is enhanced during late pregnancy and early lactation (Guesnet *et al.*, 1987). In cattle there is an increase in the response to catecholamines and an increase in the number of β -receptors on adipocytes during early lactation (Jaster & Wegner, 1981).

The factors responsible for these changes in responsiveness of adipocytes to catecholamines during the lactation cycle have not been fully resolved. Studies with rats suggest that growth hormone rather than prolactin is implicated (Vernon *et al.*, 1987). Also, prolonged treatment of cattle with recombinant growth hormone accentuates the ability of catecholamines to increase plasma fatty acid and glycerol concentrations *in vivo* (Peters, 1986; Boyd & Bauman, 1988).

In the present study we have used a tissue culture system to show that chronic exposure of sheep adipose tissue to growth hormone increases the response and sensitivity of the tissue to β -agonists, and that this is probably due to an increase in the number of β -adrenergic receptors on the adipocytes. During the course of these studies we also found to our surprise that insulin attenuates the ability of the α_2 -adrenergic system to inhibit lipolysis.

EXPERIMENTAL

Sheep were either 6–9-month-old castrated males (wethers) or 3- or 4-year-old multiparous Finn $\times$ Dorset Horn cross-bred ewes, and were fed on a diet of hay and cereals (Vernon *et al.*, 1981) for at least 1 month before slaughter. On the day of killing, sheep were anaesthetized by injection of 20–30 ml of Sagatal (May & Baker, Dagenham, Essex, U.K.) into the jugular vein, after which they were exsanguinated and samples of subcutaneous adipose tissue were removed aseptically from the flank fat-pads immediately anterior to the hind limbs. Adipose tissue was placed immediately in sterile 0.15 M-NaCl at about 37 °C.

Pieces of adipose tissue (about 10 mg) were cut with scissors and then either used immediately for measurement of the rate of lipolysis or preparation of membranes, or maintained in tissue culture for up to 48 h at 37 °C under air/CO₂ (19:1) in medium 199 with Earle's salts, 25 mM-Hepes (pH 7.3), 2.6 mM-acetate (final concentration) and antibiotics (Robertson *et al.*, 1982). The medium was supplemented with ovine growth hormone (a gift from NIAMDD, Bethesda, MD, U.S.A.) and insulin (Sigma) as described in the text and Table legends.

The rate of lipolysis (glycerol release) of tissue pieces before and after tissue culture was measured over a 3 h incubation period at 37 °C in Krebs–Ringer bicarbonate (Krebs & Henseleit, 1932) (1.22 mM-CaCl₂) supplemented with 5.5 mM-glucose and 30 mg of BSA/ml (dialysed and fatty-acid-free) as described previously (Vernon & Finley, 1985). The incubation medium was supplemented with various concentrations of either isoprenaline or noradrenaline, as indicated in the text and Table legends.

Adipocytes were prepared from pieces of fresh tissue or from tissue pieces after culture by collagenase digestion; the digestion medium was Krebs–Ringer bicarbonate buffer (1.22 mM-CaCl₂) (Krebs & Henseleit, 1932) containing 5.5 mM-glucose, 2 mM-acetate, 30 mg of fatty-acid-free BSA/ml, 25 mM-Hepes, pH 7.4, and 1.5 mg of collagenase/ml (Sigma, type II). After about 60 min at 37 °C, undigested material was removed by filtration through a nylon mesh. Adipocytes were separated from stroma-

Abbreviations used: ED₅₀, concentration causing half-maximal effect.

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vascular cells by flotation and washed twice with the digestion medium (with 10 mg of albumin/ml and no collagenase). All operations were performed at about 37 °C. Adipocytes were disrupted by suspension in 10 ml of Tris/HCl (pH 7.4)/20 mM-EDTA/0.2 M-sucrose/0.2 mM phenylmethanesulphonyl fluoride at 40 °C and vortex-mixed for 2 min at 40 °C. This preparation was immediately centrifuged, without pre-cooling of the suspension, at 42000 g for 30 min with the temperature setting at 4 °C; this minimizes trapping of membranes within the lipid layer. The membrane pellet was resuspended in 50 mM-Tris/HCl, (pH 7.4)/10 mM-MgCl₂, snap-frozen and stored in liquid N₂.

Binding of (–)-[³H]dihydroalprenolol (specific radioactivity 57 Ci/mmol), [*o*-methyl-³H]rauwolscine (specific radioactivity 83 Ci/mmol) and (–)-N⁶-[³H]phenylisopropyladenosine (specific radioactivity 53 Ci/mmol) (all obtained from Amersham International, Amersham, Bucks., U.K.) to sheep adipocyte membranes was performed in 50 mM-Tris/HCl (pH 7.4)/10 mM-MgCl₂ at 37 °C in a final volume of 0.2 ml containing 20–200 µg of membrane protein and the appropriate radioligand at a concentration of 20 nM (Malbon *et al.*, 1978). The reaction was initiated by addition of membrane suspensions. Incubations were stopped after 10 min by addition of 5 ml of ice-cold incubation buffer. The contents of each assay tube were then rapidly filtered under vacuum through Whatman GF/C filter discs (2.5 cm diameter). The filters were then washed with 2 × 5 ml of ice-cold incubation buffer and dried under vacuum. Filters were placed in 10 ml of Opti-Fluor scintillant (Packard Instrument Co.) and counted for a radioactivity on an LKB 1215 Rackbeta liquid-scintillation counter. Assays were performed in duplicate. Non-specific binding was determined by incubating corresponding tubes with 100 µM-(–)-isoprenaline, 100 µM-yohimbine or 100 µM-N⁶-phenylisopropyladenosine (to measure β-, α₂- and adenosine receptor ligand binding respectively). Specific binding of radioligands is defined as the total [³H]ligand bound minus [³H]ligand bound in the presence of 100 µM competing ligand. For binding studies using [³H]phenylisopropyladenosine, plasma membranes (20–100 µg) were first resuspended in incubation buffer (50 mM-Tris/HCl, pH 7.4, and 1 mM-MgCl₂) and incubated with adenosine deaminase (1 µg/ml) for 10 min at 30 °C (final volume of assay mix was 100 µl (Trost & Schwabe, 1981). Adenosine-deaminase-treated plasma membranes were used immediately to assay adenosine-receptor binding. Preparatory studies showed that the incubation time used was optimal for ligand binding in each case; 20 nM ligand was sufficient to saturate the binding capacity of sheep fat-cell membranes and thus to achieve essentially maximum binding both before and after tissue culture; binding was proportional to the amount of membrane added over the stated range.

5'-Nucleotidase activity of the membrane preparation was assayed as described previously (Vernon *et al.*, 1983). Protein concentration was determined by the Bradford method (Bradford, 1976) using IgG as a standard. The number of adipocytes/g of tissue was determined as described previously (Robertson *et al.*, 1982), but using the dry weight rather than the lipid weight/g wet weight of tissue for the estimation.

Results are expressed as means ± S.E.M. and were analysed using Student's *t* test for paired data.

RESULTS

Response of adipose tissue to isoprenaline and noradrenaline

Preparatory studies were performed with wethers in the autumn. These studies showed that maintenance of adipose tissue in culture for 48 h increased ($P < 0.05$) the basal rate of lipolysis from 9 ± 4 to 63 ± 10 nmol/h per 10^5 cells (three observations). The maximum response to isoprenaline was

achieved with 10 µM agonist, and the ED₅₀ (concentration giving a half-maximal effect) was 110 ± 29 nM. Tissue culture for 48 h decreased ($P < 0.05$) the ED₅₀ to 10 ± 3 nM and resulted in a small but consistent increase in the maximum response to isoprenaline. The changes in basal lipolysis and in the response and sensitivity to isoprenaline occurred mostly over the first 24 h of tissue culture, with little or no further change over the second 24 h period; thus a new steady-state had been achieved by 48 h of culture.

Possible acute effects of insulin and growth hormone were examined in further preparatory studies using 3 h incubations of fresh tissue. Neither growth hormone (4.5 nM) nor insulin (17 nM) had any effect on basal lipolysis. Growth hormone had no effect on lipolysis in the presence of 10 µM-isoprenaline or 10 µM-noradrenaline, whereas insulin decreased the lipolytic effects of 10 µM-isoprenaline and 10 µM-noradrenaline by less than 10%.

The chronic effects of growth hormone and insulin on lipolysis were investigated in two separate experiments, one performed in January–March (four sheep) and the other in May–June (five sheep) of a subsequent year (Table 1). Marked variations in the rate of lipolysis and in the size of response to treatments were found. In general, rates of catecholamine-stimulated lipolysis were highest in the first experiment, performed in January–March. High rates of catecholamine-stimulated lipolysis were less well sustained during culture than lower rates.

Maintenance of adipose tissue from ewes in culture in the absence of exogenous hormones for 48 h increased the subsequent basal rate of lipolysis ($P < 0.01$) (Table 1). The basal rate of lipolysis was further increased ($P < 0.01$) by inclusion of insulin in the culture medium. Addition of growth hormone to the culture medium had no effect on the basal rate of lipolysis (Table 1).

Tissue culture in the absence of exogenous hormones resulted in a tendency for isoprenaline-stimulated lipolysis to decrease in adipose tissue from ewes, but this was not statistically significant. Addition of growth hormone to the culture medium consistently increased the rate of lipolysis in the presence of 10 µM-isoprenaline in tissue from ewes ($P < 0.001$) (Table 1), and also in tissue from wethers ($P < 0.05$) [rates of isoprenaline-stimulated lipolysis after a 48 h culture in the absence and the presence of 4.5 nM-growth hormone were 168 ± 24 and 208 ± 31 nmol/h per 10^5 cells respectively (eight observations)]. Culture with insulin also increased the rate of lipolysis in the presence of isoprenaline in tissue from ewes ($P < 0.05$) (Table 1) and wethers ($P < 0.05$) (results not shown) to about the same level as did culture with growth hormone. However, when the basal rate of lipolysis was subtracted it was found that growth hormone markedly increased the response to isoprenaline ($P < 0.001$), whereas the effect of insulin was due solely to increased basal lipolysis (Table 1).

Tissue culture for 48 h with either insulin or growth hormone also increased the rate of lipolysis in the presence of noradrenaline ($P < 0.01$ and $P < 0.05$ respectively) (Table 1). The rate of lipolysis in the presence of noradrenaline after culture with insulin was significantly greater ($P < 0.05$) (Table 1) than that after culture with growth hormone. Subtraction of the basal rate of lipolysis confirmed that growth hormone increased the response to noradrenaline ($P < 0.01$) (Table 1). Much of the effect of insulin was again due to an increase in basal lipolysis, but, in contrast with its effects on isoprenaline-stimulated lipolysis, insulin did appear to have a small effect on the response to noradrenaline, as the increase in rate due to noradrenaline was intermediate between that found after culture without exogenous hormones and that with growth hormone (Table 1). Noradrenaline differs from isoprenaline in that it not only stimulates lipolysis acting via the β-adrenergic system, but it also has an inhibitory effect on lipolysis acting via the α₂-adrenergic system.

Table 1. Effects of tissue culture in the presence of growth hormone or insulin on the rates of basal, isoprenaline-stimulated and noradrenaline-stimulated lipolysis in adipose tissue from adult female sheep

Pieces of adipose tissue were maintained in tissue culture for 48 h in the presence and absence of either 4.5 nM-growth hormone or 17 nM-insulin, after which the rate of lipolysis was measured in the absence of catecholamines (basal rate), or in the presence of 10 μ M-isoprenaline or 10 μ M-noradrenaline as described in the text. Pooled results from two experiments are presented; one experiment was performed in January–March and the second in May–June of a subsequent year. Results are means $\pm$ S.E.M. of nine observations. Significant differences from values found after culture without exogenous hormones are indicated by * P < 0.05, ** P < 0.01 and *** P < 0.001 (analysis by Student's t test for paired observations).

Variable	Culture conditions ...	Glycerol release (nmol/h per 10 ⁵ cells)			
		Pre-culture	48 h; no additions	48 h; + growth hormone	48 h; + insulin
Basal rate (A)		12 $\pm$ 3**	67 $\pm$ 15	65 $\pm$ 20	145 $\pm$ 27**
Rate in presence of 10 μ M-isoprenaline (B)		280 $\pm$ 57	220 $\pm$ 27	285 $\pm$ 28***	280 $\pm$ 43*
Increase in rate due to isoprenaline (B–A)		268 $\pm$ 58	153 $\pm$ 17	220 $\pm$ 19***	135 $\pm$ 19
Rate in presence of 10 μ M-noradrenaline (C)		230 $\pm$ 63	142 $\pm$ 21	205 $\pm$ 35*	254 $\pm$ 39**
Increase in rate due to noradrenaline (C–A)		218 $\pm$ 63	75 $\pm$ 7	140 $\pm$ 16**	100 $\pm$ 21
100 $\times$ ratio of rate in presence of 10 μ M-noradrenaline/10 μ M-isoprenaline (C/B)		75.9 $\pm$ 6.4	64.8 $\pm$ 4.3	70.0 $\pm$ 6.0	91.8 $\pm$ 4.3**

The difference between the rates of noradrenaline and isoprenaline-stimulated lipolysis provides a measure of the α_2 -adrenergic effect of noradrenaline (Rebuffé-Scrive *et al.*, 1989). A corollary of this is that a change in the ratio of the rate of noradrenaline-stimulated/isoprenaline-stimulated lipolysis reflects a change in α_2 -adrenergic response to noradrenaline. Addition of insulin to the culture medium consistently increased the ratio of noradrenaline-stimulated/isoprenaline-stimulated lipolysis (P < 0.01) (Table 1), indicating a substantial suppression of the α_2 -adrenergic effect of noradrenaline.

Sensitivity of adipose tissue to isoprenaline

Addition of growth hormone to the culture medium increased the sensitivity to isoprenaline; this was observed in two individual experiments with ewes (mean ED₅₀ values after culture with and without growth hormone were 6.3 and 19 nM) and in a study with four wethers (mean ED₅₀ values after culture with and without growth hormone were 0.55 and 2.1 nM). Analysis of log-transformed results for all six sheep confirmed this effect to be significant (P < 0.05). Tissue culture with growth hormone also increased (P < 0.05) sensitivity to noradrenaline in adipose tissue from ewes (mean ED₅₀ values after culture with and without growth hormone were 0.24 $\pm$ 0.02 and 0.35 $\pm$ 0.02 μ M respectively).

Ligand binding to adrenergic and adenosine receptors

Ligand binding to receptors was performed with a single concentration of ligand in each case. Separate experiments using membranes from tissue both before and after culture confirmed that this concentration was sufficient to saturate the specific component of ligand binding in each case. Tissue culture in the absence of exogenous hormones had no effect on ligand binding to β -receptors in sheep adipose tissue. Inclusion of growth hormone, but not insulin, in the culture medium significantly increased binding (P < 0.05) (Table 2). Addition of insulin to the medium partly diminished the effect of growth hormone (Table 2).

Tissue culture alone resulted in a substantial increase in ligand binding to the α_2 -receptor (P < 0.05) (Table 2), but had no effect

Table 2. Effects of tissue culture for 48 h in the presence and absence of growth hormone and/or insulin, on ligand binding to the β -, α_2 - and adenosine receptors of adipocyte membrane fractions from adult female sheep

Pieces of adipose tissue were maintained in culture for 48 h as described in the text with or without growth hormone (4.5 nM) and/or insulin (17 nM). Subsequently, adipocytes were prepared by collagenase digestion, disrupted by agitation in a hypo-osmotic solution, and a membrane fraction was prepared by centrifugation as described in the text. Binding of 20 nM-[³H]dihydroalprenolol, 20 nM-[³H]rauwolscine and 20 nM-[³H]N⁶-phenylisopropyladenosine to β -, α_2 - and adenosine receptors respectively was measured as described in the text. Results are means $\pm$ S.E.M. of six observations in each case. Significant differences from values after culture with no additions are indicated by * P < 0.05 and ** P < 0.01 (using Student's t test for paired results).

Culture conditions	Ligand binding (fmol/mg of protein)		
	β -Receptor	α_2 -Receptor	Adenosine receptor
Pre-culture	28.0 $\pm$ 5.1	5.5 $\pm$ 2.4*	32.5 $\pm$ 12.5
Culture for 48 h; no additions	33.1 $\pm$ 7.0	25.3 $\pm$ 7.7	44.0 $\pm$ 19.8
Culture for 48 h; + growth hormone	51.6 $\pm$ 10.2**	25.5 $\pm$ 5.7	47.0 $\pm$ 19.9
Culture for 48 h; + insulin	30.9 $\pm$ 6.2	12.1 $\pm$ 4.8*	34.8 $\pm$ 15.4
Culture for 48 h; + growth hormone + insulin	37.9 $\pm$ 7.8	15.5 $\pm$ 5.5	27.3 $\pm$ 8.0

on the affinity for the ligand (results not shown). Inclusion of growth hormone had no effect on ligand binding to the α_2 -receptor, whereas insulin partly prevented the increase in ligand binding during culture [the value after culture with insulin was significantly less (P < 0.05) than after culture alone]. The factors responsible for the increase in ligand binding to α_2 -receptors during culture are not known. An increase in the number of α_2 -

adrenergic receptors seems probable, as studies with wethers showed that the increase in ligand binding could be prevented by inclusion of 80 nm-actinomycin D in the culture media; mean [^3H]rauwolscine binding was 18.8, 73.5 and 13.9 fmol/mg of protein for adipocyte membranes from tissue before culture, after culture for 48 h and after culture for 48 h in the presence of actinomycin D (results are means of four observations; standard error of difference was 7.5; $P < 0.001$ for actinomycin D effect). Medium 199 contains Phenol Red, which has weak oestrogenic properties (Berthois *et al.*, 1986); however, neither culture in Phenol Red-free medium 199 nor inclusion of an anti-oestrogen (tamoxifen) in the culture medium prevented the increase in ligand binding to the α_2 -receptors (results not shown).

Neither tissue culture alone nor addition of growth hormone or insulin to the media had any effect on ligand binding to the adenosine receptor (Table 2) or on 5'-nucleotidase activity of the membranes (results not shown).

DISCUSSION

This study shows that chronic endocrine control of the adrenergic system of sheep adipose tissue can be achieved in tissue culture. Marked variations in rates of lipolysis and sizes of responses to treatment were observed between individual animals, probably because sheep are less inbred than, e.g., laboratory rats. In addition, the lipolytic rate and the size of the response appear to vary with season; seasonal changes in adipose tissue metabolism are known to occur in ruminants (Larsen *et al.*, 1985; Vernon & Taylor, 1986; Vernon *et al.*, 1986).

One clear and consistent observation which emerges is that chronic exposure to growth hormone increases the response and sensitivity of sheep adipose tissue to β -agonists. Furthermore, this effect is at least partly due to an increase in ligand binding by β -adrenergic receptors which, since a saturating concentration of ligand was used, probably reflects an increase in the number of β -adrenergic receptors per adipocyte. Other studies have shown that increasing serum growth hormone concentrations *in vivo* enhances the lipolytic response to catecholamines in cattle, as judged by changes in serum glycerol and non-esterified fatty acid concentrations after injection of catecholamines (Peters, 1986; Boyd & Bauman, 1988). In addition, manipulating serum growth hormone concentrations in the rat also results in a change of response to catecholamines in isolated adipocytes (Vernon *et al.*, 1987). The present study thus extends these observations by showing that growth hormone can act directly on adipose tissue to elicit such effects, and that the mechanism probably involves an increase in the number of β -adrenergic receptors. As the serum growth hormone concentration is usually increased during lactation in ruminants (see Bauman & Elliot, 1983; Forsythe, 1986), and the number of β -adrenergic receptors on adipocytes is also increased (Jaster & Wegner, 1981), it is probable that the increased response (Jaster & Wegner, 1981; Vernon & Finley, 1985; Guesnet *et al.*, 1987) and sensitivity (Guesnet *et al.*, 1987) of ruminant adipose tissue to β -agonists during lactation is at least partly due to a growth hormone-induced increase in the number of β -receptors.

Although it has been known for many years that treatment of animals with growth hormone leads to a loss of body fat, the mechanism of this effect remains unresolved. There is still a continuing controversy as to whether growth hormone itself has a direct lipolytic effect in adipocytes, which has not been resolved despite the availability of ultrapure recombinant growth hormone

(see Boyd & Bauman, 1988; Vernon & Flint, 1989). The present study supports the view that at least some of the effects of growth hormone on adiposity may be indirect, arising through a change in the responsiveness of adipose tissue to catecholamines.

The acute anti-lipolytic effects of insulin are well documented in laboratory species and are also found with ruminant adipose tissue (see Vernon, 1980), so that chronic effects of insulin on the adipocyte adrenergic system were unexpected in that they predispose towards higher rates of lipolysis. The effects of insulin are distinct from those of growth hormone; the former appears to diminish signal transduction via the α_2 -adrenergic system, as indicated by the increase in the ratio of noradrenaline-stimulated/isoprenaline-stimulated lipolysis. The mechanism is unclear, but may involve an effect on the number of α_2 -adrenergic receptors. However, other mechanisms may also be involved; for example, a defect at the level of the interaction of the guanine-nucleotide-binding protein G_i with adenylate cyclase could account for the increase in basal lipolysis found after culture with insulin as well as the diminished activity via the α_2 -adrenergic system.

We thank Miss S. Lindsay for technical assistance, and Mr. C. E. Park and Mr. D. Fullarton for care of the sheep.

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