Growth hormone decreases the response to anti-lipolytic agonists and decreases the levels of G_{i2} in rat adipocytes

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The effect of growth hormone (GH) *in vivo* on the G_i-mediated anti-lipolytic signalling system of rat adipocytes has been investigated. Lowering of serum GH levels, by treatment of rats with an antiserum (anti-rGH) specific for rat GH, increased the sensitivity of adipocytes to the anti-lipolytic agonists *N*⁶-phenylisopropyladenosine (PIA) and prostaglandin E₁. This occurred in the absence of any change in PIA binding to adipocyte membranes. Immunoblot analysis of adipocyte membranes

revealed that lowering of serum GH resulted in at least a 3-fold increase in the levels of α -subunit of G_{i2}, but had no effect on the α -subunits of G_{i1} and G_{i3} nor on the 42 and 45 kDa forms of the α -subunit of G_s. Replenishment of serum GH, by concurrent administration of ovine GH to rats, prevented all of these effects of anti-rGH. It is concluded that GH down-regulates the amount of G_{i2} α -subunit in adipocyte membranes, resulting in a decrease in the sensitivity of the cells to anti-lipolytic agonists.

INTRODUCTION

Adipocyte lipolysis is under acute regulatory control from both stimulatory and inhibitory agonists. The rate-limiting enzyme, hormone-sensitive lipase, can be phosphorylated and activated by cyclic-AMP-dependent protein kinase. Therefore changes in adenylate cyclase (AC) activity, which modulate cyclic AMP levels, are intimately connected with the control of lipolysis (Belfrage, 1985).

AC activity can be regulated by both stimulatory (e.g. glucagon and β -adrenergic) and inhibitory (e.g. adenosine and prostaglandin) receptors which exert their actions through the heterotrimeric G-proteins, G_s and G_i respectively. Each of these G-proteins comprises an α -subunit, together with β - and γ -subunits: the α -subunit couples to the appropriate receptors as well as to the catalytic subunit of AC (Levitzki, 1990). Thus G_i, through either direct action of its α -subunit on AC or, by release of β -subunits which serve to attenuate the dissociative activation of α -G_s, induces inhibition of AC (Levitzki, 1990; Birnbaumer, 1992; Tang and Gilman, 1992).

Changes in the expression of adipocyte G_i and G_s have been shown to occur in a number of animal models of pathological states, such as chemically induced hypothyroidism (Milligan and Saggerson, 1990), genetic obesity (Strassheim et al., 1991; Bégin-Heick, 1992) and insulin resistance (Strassheim et al., 1990, 1991; Saggerson et al., 1991; Bégin-Heick, 1991), and also in obese humans (Ohisalo and Milligan, 1989). In a number of such instances these changes have been shown to correlate with impaired functioning of agonists which regulate lipolysis (Ohisalo and Milligan, 1989; Milligan and Saggerson, 1990; Strassheim et al., 1991; Bégin-Heick, 1992).

In several species, including the rat, growth hormone (GH) has been shown to decrease adiposity through changes in the rate of both lipid synthesis and also lipolysis (Goodman et al., 1988; Vernon and Flint, 1989; Cuneo et al., 1992; Flint and Gardner, 1993). Recent studies suggest that GH effects on lipolysis are indirect and are achieved by altering the maximal response or sensitivity of adipocytes to acutely acting lipolytic or anti-

lipolytic agents. Treatment of animals with GH increases the lipolytic response to catecholamines *in vivo* (Sechen et al., 1990), and, in some circumstances, *in vitro*; litter removal from lactating rats results in a fall in maximum response to catecholamines, which is prevented by treatment with GH (Vernon et al., 1993). In other states treatment with GH appears to have little effect on response to catecholamines *in vitro*, but it does diminish the response (Vernon et al., 1987, 1991) and/or sensitivity (Lanna et al., 1992) to the anti-lipolytic agent adenosine. This has prompted us to investigate whether GH can influence the regulation of lipolysis by altering the expression of G_i and G_s in rat adipocytes, and how this may correlate with the functioning of inhibitory receptors.

EXPERIMENTAL

Female Wistar rats (200–250 g; A. Tuck and Son, Rayleigh, Essex, U.K.) were fed on Labsure irradiated CRM diet (Labsure, Poole, Dorset, U.K.) and water *ad libitum*. To minimize stress, animals were accustomed to handling before the commencement of experimentation. Injections were administered twice daily, at 09:00 and 17:00 h, for 2 days. Rats received the following, as specified in legends: γ -globulin fraction of antiserum to rat GH (anti-rGH; 150 mg/injection, equivalent to 4.5 ml of serum), either alone or in combination with ovine GH (NIADDK-oGH-13), 0.5 mg per injection. Control animals received carrier solutions (Madon et al., 1986). Anti-rGH, which was generously provided by Dr. D. J. Flint, Hannah Research Institute, is specific for rat GH and does not recognize oGH. Details of its preparation and characterization have been described previously (Madon et al., 1986). On day 3, between 10:00 and 11:00 h, rats were anaesthetized as previously described (Vernon et al., 1993).

Immediately after the induction of anaesthesia, samples of blood and parametrial adipose tissue were taken, after which rats were killed by exsanguination. There were ten rats in each treatment group; five were used for measurement of lipolysis and five for preparation of membranes.

Abbreviations used: AC, adenylate cyclase; GH, growth hormone; IGF-1, insulin-like growth factor-1; anti-rGH, antiserum to rat GH; oGH, ovine GH; PGE₁, prostaglandin E₁; PIA, *N*⁶-phenylisopropyladenosine.

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Preparation of serum and adipocytes

Serum was prepared and stored at -20°C for assay of insulin-like growth factor-1 (IGF-1) (Madon et al., 1986).

Adipocytes were prepared by digestion with 1 mg/ml collagenase (Sigma type II) in M199* [medium 199 containing Earle's salts, 2 mM L-glutamine, 5.5 mM glucose, 25 mM Hepes, pH 7.3, and 3% (w/v) dialysed and fatty-acid-free BSA], as described previously (Vernon et al., 1993). After digestion, adipocytes were washed three times with M199* before measurement of lipolysis or preparation of membranes.

Adipocyte size and the number of cells/ml were determined as described previously (Vernon et al., 1993).

Membrane preparation

Adipocyte membranes were prepared as described previously (Strassheim et al., 1991). Membranes were stored in liquid N_2 until required for immunoblotting or for measurement of ligand binding. Protein content was determined by the Bradford (1976) method with BSA (Sigma, fraction V) as a standard.

Immunoblotting

Anti-peptide antisera CS1, IIC and SG1, which specifically recognize the α -subunits of the G-proteins G_s , G_{i1} and G_{i2} respectively, were kindly given by Dr. G. Milligan, Department of Biochemistry, University of Glasgow (Mitchell et al., 1989; Strassheim et al., 1990). An anti-peptide antiserum which recognizes amino acids 345–354 of the α -subunit of G_{i3} was purchased from Calbiochem (Nottingham, U.K.).

Membrane samples were resolved by SDS/PAGE on 12%-acrylamide gels (7.5 cm-length mini-gels for detection of G_i and 20 cm-length gels for detection of G_s). Immunoblotting was performed essentially as described previously (Mitchell et al., 1989; Strassheim et al., 1990, 1991). Briefly, proteins were transferred to nitrocellulose, and then blocked overnight at room temperature with 2.5% (w/v) skimmed milk (Marvel). Primary antiserum, in 1% Marvel, was then added for 4 h. After removal of primary antiserum, the blot was washed extensively, and then incubated with ^{125}I -labelled Protein A (Amersham International, Amersham, Bucks., U.K.) for the detection of immunoreactive proteins. Quantification of reactive proteins was achieved by γ -radiation counting of excised bands (for G_{i1} , G_{i2} and G_s) or by densitometric scanning using a Bio-Rad 620 video densitometer (for G_{i3}).

Control experiments established that for each antiserum the intensity of labelling was proportional to the amount of membrane protein (2–20 μg for G_i and 25–75 μg for G_s).

Rate of lipolysis

The rate of lipolysis (glycerol release) of isolated adipocytes was determined as described previously (Vernon et al., 1987). Cells were incubated in M199* supplemented with 100 nM isoprenaline, 0.8 $\mu\text{g}/\text{ml}$ adenosine deaminase and the indicated concentrations of N' -phenylisopropyladenosine (PIA) or prostaglandin E_1 (PGE_1), and the amount of glycerol released during incubation for 60 min at 37°C was determined.

Ligand binding to adenosine receptors

This was performed with $[^3\text{H}]\text{PIA}$, as described previously (Watt et al., 1991), with 56 μg of membrane protein per assay tube and

with either only 20 nM or a range of $[^3\text{H}]\text{PIA}$ concentrations (1–100 nM).

Analysis of results

Statistical significance was assessed by analysis of variance. Unless otherwise stated results are given as means $\pm$ SED (standard error of difference).

RESULTS

Serum IGF-1

Rats were treated with anti-rGH to decrease serum GH levels. In addition to anti-rGH, some animals also received oGH; these animals served as a control against non-specific effects of the antiserum. Effectiveness of the anti-rGH, and also of injections of oGH, were checked by assaying for serum IGF-1. Treatment of rats with anti-rGH alone significantly ($P < 0.001$) decreased the serum IGF-1 concentration, as compared with that in control animals, and concurrent injection of oGH prevented this decrease. Serum IGF-1 levels were 496 ng/ml for control animals, 233 ng/ml for anti-rGH-treated animals and 644 ng/ml for anti-rGH + oGH-treated animals (SED = 51, $n = 11$ animals in each case).

Lipolytic response to PIA and PGE_1

Maximal stimulation of lipolysis was achieved, as reported previously (Vernon et al., 1987), by including 0.8 $\mu\text{g}/\text{ml}$ adenosine deaminase and 100 nM isoprenaline in incubations. Treatment of rats with anti-rGH, either with or without oGH, had no effect either on basal lipolysis or on the stimulation of lipolysis by isoprenaline. Basal glycerol release, measured in the presence of adenosine deaminase, was 0.5 ± 0.1 $\mu\text{mol}/\text{min}$ per 10^6 cells, and 100 nM isoprenaline increased glycerol release to 5.4 ± 0.1 $\mu\text{mol}/\text{min}$ per 10^6 cells (results are means $\pm$ S.E.M. of 15 observations).

Figure 1 Effect of GH *in vivo* on inhibition of isoprenaline-stimulated lipolysis by PIA

Adipocytes were prepared from rats injected with anti-rGH, with (Δ) or without oGH (■), or carrier solutions (○). Glycerol release was measured, in the presence of 0.8 $\mu\text{g}/\text{ml}$ adenosine deaminase, 100 nM isoprenaline and the indicated concentration of PIA, as described in the Experimental section. Results are means of five observations, and the SED for comparing values at the same PIA concentration is 10.15. The SED value was determined by analysis of variance of all values.

Table 1 Effect of GH *in vivo* on inhibition of isoprenaline-stimulated lipolysis by PGE₁

Adipocytes were prepared from rats injected with anti-rGH, with or without oGH, or carrier solutions. Glycerol release was measured, in the presence of the indicated concentrations of PGE₁, as described in the legend to Figure 1. Results are means of five observations and the SED, determined by analysis of variance of all values, for comparing values in the same column is 7.3; ^a and ^b indicate that the value is significantly different from the appropriate value for rats which received carrier solutions ($P < 0.02$) or anti-rGH ($P < 0.01$) respectively.

Injection	PGE ₁ concn. ...	Glycerol release (% inhibition)	
		100 nM	1 μ M
Carrier solutions		50.6	70.4
Anti-rGH		70.4 ^a	78.8
Anti-rGH, oGH		40.0 ^b	64.0

Lowering of serum GH levels by injecting rats with anti-rGH resulted in a significant increase ($P < 0.05$) in the inhibition of lipolysis induced by both 3 nM and 10 nM PIA (Figure 1). This effect was prevented by concurrent injection with oGH. In contrast, anti-rGH had no significant effect on the inhibition of lipolysis by 100 nM PIA (Figure 1). Treatment with anti-rGH also significantly increased ($P < 0.02$) the inhibition of lipolysis observed with 100 nM PGE₁ (Table 1). As described above, this effect was prevented by concurrent treatment with oGH. Although anti-rGH induced a numerical increase in the inhibition of lipolysis by 1 μ M PGE₁, this action was not statistically significant (Table 1).

These results demonstrate that GH suppresses the anti-lipolytic effects of PIA and PGE₁, by decreasing the sensitivity of

adipocytes to sub-maximal concentrations of these agents. However, GH appears to have little effect on the response of lipolysis to maximally effective concentrations of either PIA or PGE₁.

Ligand binding to adenosine receptors

Initially, [³H]PIA binding was measured by using a single concentration (20 nM); the amount bound was 83.0, 96.7 and 71.0 fmol/mg of protein for control, anti-rGH-treated and anti-rGH + oGH-treated rats respectively (results are means of 5 observations; SED was 15.5 fmol/mg of protein, indicating no significant effect of treatments). A subsequent experiment was performed using a range of [³H]PIA concentrations (Table 2). Maximal binding was achieved with about 100 nM [³H]PIA. No significant effect of manipulation of serum GH levels was observed with any concentration of [³H]PIA used.

Immunoblotting of α -subunits of G₁, G₂, G₃ and G_s in adipocyte membranes

Anti-peptide antisera were used to ascertain changes in the levels of α -subunits of individual G-proteins in adipocyte membranes from control, anti-rGH-treated and anti-rGH + oGH-treated animals. Results are expressed in arbitrary units (Table 3). It should be noted that, as we do not know the affinities of the different antisera, we are unable to comment on the absolute amounts of G α subunits in the membranes.

Rat adipocytes express three forms of G_i (G_{i1}, G_{i2} and G_{i3}), which are distinct gene products (Milligan, 1988; Mitchell et al., 1989). They also express two forms of G_s (42 kDa and 45 kDa), derived by alternative splicing (Robishaw et al., 1986; Strassheim et al., 1990, 1991). Injection of rats with anti-rGH, either alone or in the combination with oGH, had no effect on the expression of the α -subunit of G_{i3} (α -G_{i3}) or on the 45 kDa and 42 kDa α -subunit forms of G_s (Figure 2, Table 3). The similarity in size of

Table 2 Effect of varying the serum GH concentration on the ability of adipocyte membranes to bind [³H]PIA

Rats were injected twice daily for 2 days with carrier solution, anti-rGH or anti-rGH plus oGH, after which adipocyte membranes were prepared, and their ability to bind concentrations of [³H]PIA (1 to 100 nM) was assessed as described in the Experimental section. Each value is the mean of 3 separate observations (adipocytes from 2 rats were pooled for each preparation); pooled SED values were derived from analysis of variance, values for each concentration of PIA being analysed separately.

Treatment	[PIA] (nM) ...	[³ H]PIA bound (fmol/mg of protein)								
		1	2	4	6	8	10	20	50	100
Carrier solutions		13.33	26.19	42.86	64.17	75.48	85.71	125.24	240.48	266.43
Anti-rGH		6.78	14.76	28.81	39.28	55.12	84.76	103.21	227.62	254.17
Anti-rGH, oGH		4.52	13.57	22.62	35.24	48.21	47.50	82.74	205.48	232.26
SED		5.22	10.95	17.68	22.73	29.13	32.82	41.25	46.81	50.00

Table 3 Expression of G-protein α -subunits in adipocyte membranes

Adipocyte membranes were prepared from rats which had received the indicated injections. Levels of α -subunits of G-proteins, detected by immunoblotting, are given in arbitrary units (see the experimental section). Results are means of 5 observations. Pooled SED values were derived by analysis of variance. ^a and ^b indicate that the value is significantly different ($P < 0.001$) from the value for animals treated with carrier solutions or anti-rGH respectively.

Injections	G-protein ...	G _{i1}	G _{i1} + G _{i2}	G _{i3}	G _s (45 kDa)	G _s (42 kDa)
Carrier solutions		189	33	0.89	3503	3314
Anti-rGH		233	911 ^a	0.88	3488	3842
Anti-rGH, oGH		173	206 ^b	0.76	3255	3527
SED		68	110	0.3	407	608

Figure 2 Immunoblotting of G-protein α -subunits in adipocyte membranes

(a) Detection of α -G_{i1} with antiserum I1C (10 μ g per track). (b) Detection of α -G_{i1} plus α -G_{i2} with antiserum SG1 (2.5 μ g per track). (c) Detection of α -G_{i3} (10 μ g per track). (d) Detection of the 42 kDa and 45 kDa forms of α -G_s with antiserum CS1 (30 μ g per track). Adipocyte membranes were prepared from rats injected with (1) carrier solutions, (2) anti-rGH plus oGH or (3) anti-rGH alone. Immunoblotting was performed as described in the Experimental section. Data shown are typical of experiments done five times using membranes from different rats.

the α -subunits of G_{i1} (α -G_{i1}; 41 kDa) and G_{i2} (α -G_{i2}; 40 kDa) did not permit their separation under the electrophoresis conditions used. Hence antiserum SG1, which recognizes α -G_{i1}, plus α -G_{i2}, detected only one immunoreactive band, presumably representing both α -subunits. Injection of rats with anti-rGH resulted in a 3 ± 0.27 -fold increase ($P < 0.001$; mean $\pm$ S.E.M. for 5 observations) in the amount of α -G_{i1} plus α -G_{i2}, as detected by antiserum SG1, in adipocyte membranes (Figure 2; Table 3). This increase was prevented by concurrent administration of oGH. Immunoblotting with antiserum I1C, which recognises α -G_{i1} only, demonstrated that anti-rGH had no effect on the amount of this α -subunit (Figure 2; Table 3). Hence we can conclude that the increase in intensity of the immunoreactive band, revealed by antiserum SG1, after injection of rats with anti-rGH, can be attributed entirely to an increase in α -G_{i2}. This suggests that the increase in G_{i2}, elicited by depletion of endogenous rat GH, must be over 3-fold. These results demonstrate that GH specifically and profoundly suppresses the amount of α -G_{i2} in adipocyte membranes.

DISCUSSION

The effectiveness of treatments in changing serum GH concentration was assessed by measuring changes in growth rate (results not shown) and serum IGF-1 concentrations. Measurement of serum GH itself is impractical, as the hormone is secreted in a highly pulsatile manner (Jansson et al., 1985) and the anti-(rat GH) serum interferes with the radioimmunoassay. The changes in both serum IGF-I and growth rates indicate that significant changes in serum GH were being achieved, as found in previous studies using this antiserum and GH-therapy regime (Flint and Gardner, 1993; Vernon et al., 1993).

This study provides further evidence for GH modulating lipolysis by altering the ability of acutely acting factors to influence the process. Furthermore, the study suggests that a major effect of GH is on the anti-lipolytic signalling system which operates via G_i. Indeed, in some states this may be the only effect of GH on the system as a whole, for in the present study,

using non-lactating rats, GH treatment failed to alter maximum response to β -agonists. GH also failed to increase response to β -agonists in lactating rats (Vernon et al., 1991) and cows (Lanna et al., 1992). However, GH can affect the response to β -agonists in some states: litter removal from lactating rats (Vernon et al., 1991, 1993) or cessation of lactation by removal of both serum prolactin and GH (Barber et al., 1992) leads to a substantial fall in the response to β -agonists which is prevented by concurrent treatment with GH (but not prolactin).

GH also markedly suppressed the response to PIA in lactating rats in which lactation was abruptly and completely curtailed either by litter removal (Vernon et al., 1987) or by endocrine manipulation (Barber et al., 1992). In contrast, varying serum GH, by either GH injections or injections of the anti-rGH, had only a small effect on response to maximal concentrations of PIA in normally lactating rats (Vernon et al., 1987) and lactating cows (Lanna et al., 1992), whereas in the latter it increased sensitivity to sub-maximal concentrations of PIA, as in the present study. These various observations suggest that GH can have a spectrum of effects on the G_s-G_i-based signalling systems which regulate lipolysis, but in the non-lactating animal, and seemingly in the normally lactating animal, the predominant effect is on sensitivity to anti-lipolytic agents which act via G_i. One possibility, a change in receptor number, can be excluded for adenosine at least, as our studies show that varying serum GH had no effect on [³H]PIA binding, indicating no change in the number or affinity of adenosine receptors.

That manipulation of endogenous GH levels altered the sensitivity of lipolysis to both PIA and PGE₁ (Figure 1; Table 1), agonists which couple to distinct receptors, in itself suggests that GH modifies a post-receptor step common to both agents. As both receptors presumably exert inhibitory actions on AC by coupling to G_i, changes in this G-protein could account for changes in anti-lipolytic effects. Here, our immunological analysis of adipocyte membranes revealed that treatment of animals with anti-rGH enhanced, by more than 3-fold, the amount of α -G_{i2} while having little effect on the amounts of α -G_{i1}, α -G_{i3} and α -G_s. Furthermore, the increase in α -G_{i2} was specifically prevented by replacement of endogenous GH with oGH, a treatment which in itself did not alter levels of other G-proteins. Hence we conclude that GH down-regulates the levels of α -G_{i2} in adipocyte membranes, thus providing an explanation for the decreased sensitivity of adipocytes to anti-lipolytic agonists. Certainly it is clear that GH does not achieve this by altering levels of G_s and hence affecting the stimulatory input into the system. Instead, GH appears to affect a specific form of the inhibitory G-protein family, this being G_{i2}, which various investigations have suggested is the G_i species responsible for the inhibition of AC in physiological systems (Simonds et al., 1989; McKenzie and Milligan, 1990). Thus our data imply that both the adenosine and PGE₁ receptors exert inhibitory effects by coupling to G_{i2}, although we cannot rule out the possibility that they may also interact with G_{i1} and/or G_{i3}. The similar maximal degree of inhibition of lipolysis by PIA and PGE₁, achieved in adipocytes from control and GH-deficient rats (Figure 1; Table 1), could be explained if the receptors for these agonists couple to a pool of G_{i2} which, in itself, is of sufficient size, in both groups of animals to achieve maximal inhibition of lipolysis.

It has been reported that, in rats, hypophysectomy increased the intensity of a 40 kDa band labelled by pertussis-toxin-catalysed ADP-ribosylation (Goodman et al., 1988). The 40 kDa band in this case could have represented the α -subunits of G_{i1}, G_{i2}, G_{i3} and G_o, all of which are expressed in rat adipocytes and exhibit similar molecular sizes of about 40 kDa (Milligan, 1988; Mitchell et al., 1989). The hypophysectomized rat has a number

of endocrine deficiencies, which may account for changes in G-protein expression, and this, coupled with the inability to resolve ribosylated forms of G_i/G_o on gels, and also the fact that changes in dissociation of G-protein subunits can alter their ability to be ADP-ribosylated by pertussis toxin, makes interpretation of such data very difficult. Thus our approach, using anti-rGH to deplete GH specifically, and oGH to replete it, suggests that GH deficiency is a principal cause of this change.

In conclusion, we have demonstrated that GH, *in vivo*, specifically suppresses the levels of α - G_{i2} in rat adipocytes, leading to an attenuated response to anti-lipolytic agents. This will contribute to the ability of GH to decrease body fat mass. Interestingly, in several animal models of genetically acquired obesity (Strassheim et al., 1991; Bégin-Heick, 1992) and in obese humans (Ohisalo and Milligan, 1989) adipocyte levels of G_i and G_s are lowered. In view of our study, too, this suggests that modulation of G-protein expression in adipocytes can lead to profound changes in body fat composition.

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