The insulin-like effect of growth hormone on insulin-like growth factor II receptors is opposed by cyclic AMP

Evidence for a common post-receptor pathway for growth hormone and insulin action

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The counter-regulatory effects of β -adrenergic stimulation and cyclic AMP on the insulin-like action of growth hormone (GH) on the subcellular distribution of insulin-like growth factor II (IGF-II) receptors were studied in fat cells from hypophysectomized (Hx) and sham-operated rats. For comparison, the effect of insulin on this process was also studied. Basal IGF-II binding was increased by approx. 2-fold in cells from Hx as compared with sham-operated animals. The stimulatory effect of insulin was decreased in Hx cells, mainly due to a basal redistribution but also to a reduced total number of receptors. GH exerted an acute insulin-like effect in cells from Hx rats and stimulated the translocation of IGF-II receptors from an intracellular pool to the plasma membrane. β -Adrenergic stimulation with isoprenaline or addition of the non-metabolizable cyclic AMP-analogue N^6 -monobutyl cyclic AMP induced a cellular resistance to both GH and insulin and also reduced the responsiveness to these hormones. Adenosine exerted a modulatory effect on both hormones. Binding of 125 I-labelled GH to its receptors was not significantly changed by any of these factors. It is concluded that: (1) β -adrenergic stimulation and cyclic AMP induce a cellular GH resistance at a level distal to the GH-binding site, and (2) the insulin-like effect of GH shares a common pathway with insulin which occurs at the post-binding level.

INTRODUCTION

Growth hormone (GH) has been shown to elicit a number of different insulin-like effects in rat fat cells. These effects include most of the known actions of insulin on glucose uptake and oxidation, as well as the anti-lipolytic effect (Birnbaum & Goodman, 1976; Goodman, 1981; Goodman & Coiro, 1981; Griching *et al.*, 1983; Gause *et al.*, 1983, 1985; Björge *et al.*, 1984; Schwartz & Carter-Su, 1988). The ability of GH to rapidly stimulate amino acid transport and protein synthesis is also attributed to its insulin-like action (Cameron *et al.*, 1988). The physiological significance of the insulin-like effects of GH is unclear since they can only be reproduced *in vitro* following preincubation in the absence of GH (Goodman & Coiro, 1981) or in cells from hypophysectomized (Hx) rats (Gause *et al.*, 1983). However, experiments in man have provided evidence for a rapid and important insulin-like effect of GH *in vivo* (Davidson, 1987).

In addition to its metabolic effects, insulin has also been shown to increase the binding of insulin-like growth factor II (IGF-II) to rat adipocytes (Zapf *et al.*, 1978; King *et al.*, 1980; Oppenheimer *et al.*, 1983). Detailed studies by Wardzala *et al.* (1984) showed that when receptor cycling was stopped by ATP depletion, the increased IGF-II binding was completely accounted for by an increased number of binding sites rather than by a change in affinity. Subcellular fractionation experiments also showed that insulin increases the number of binding sites through the translocation of IGF-II receptors from a large intracellular pool to the plasma membrane (Wardzala *et al.*, 1984).

We have shown previously that GH is also able to rapidly increase the number of cell-surface IGF-II receptors in adipocytes from Hx rats without changing the receptor affinity (Lönnroth *et al.*, 1987a). This effect of GH displayed may similarities with

that of insulin, but clear differences were also found with respect to kinetics and magnitude.

The effect of insulin on both glucose transport and translocation of IGF-II receptors to the plasma membrane is opposed by cyclic AMP and β -adrenergic stimulation (Taylor *et al.*, 1976; Kashiwagi *et al.*, 1983; Green, 1983; Kirsch *et al.*, 1983; Kuroda *et al.*, 1987; Lönnroth *et al.*, 1987b,c). There is evidence that the inhibition of insulin action is exerted partly through decreased insulin binding (Kirsch *et al.*, 1983; Lönnroth & Smith, 1983; Pessin *et al.*, 1983) and partly through an interaction with an early post-binding event, including attenuation of the receptor tyrosine kinase activity (Stadtmauer & Rosen, 1986; Häring *et al.*, 1986). In the present study we have characterized the interaction between GH and cyclic AMP in order to elucidate the possibility that the insulin-like effect of GH and the effect of insulin are exerted through similar mechanisms subsequent to the binding of the hormones to their receptors.

MATERIALS AND METHODS

Materials

Human 125 I-labelled IGF-II and unlabelled IGF-II were purified from serum as previously reported (Enberg *et al.*, 1984) and were generously supplied by Dr. K. Hall and Dr. M. Tally, Karolinska Hospital, Stockholm, Sweden. Human recombinant IGF-II was purchased from Bissendorf Biochem, Hannover, Germany. Crystalline insulin was kindly donated by the Eli Lilly Co., Indianapolis, IN, U.S.A., and human recombinant growth hormone (hGH) was donated by Kabi Vitrum AB, Stockholm, Sweden. Cortisol was purchased from Upjohn, Kalamazoo, MI, U.S.A., and collagenase, isoprenaline, N^6 -monobutyl cyclic AMP (N^6 -mbcAMP), KCN, bovine serum albumin, protease inhibitors and L-thyroxine were from Sigma Chemical Co.,

Abbreviations used: (h)GH, (human) growth hormone; Hx, hypophysectomized; IGF-II, insulin-like growth factor II; N^6 -mbcAMP, N^6 -monobutyl cyclic AMP; ADA, adenosine deaminase; PIA, N^6 -(*R*-phenylisopropyl)adenosine; EC₅₀, concn. causing 50% of maximal effect.

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St. Louis, MO, U.S.A. Medium 199 was obtained from Statens Bakteriologiska Laboratorium, Stockholm, Sweden. Adenosine deaminase (ADA) and *N*⁶-(*R*-phenylisopropyl)adenosine (PIA) were purchased from Boehringer, Mannheim, Germany.

Animals

Male Sprague-Dawley rats (140–170 g) (Alab, Stockholm, Sweden) were either hypophysectomized according to standard procedures or sham-operated (Smith, 1930). Hx rats were injected subcutaneously with 2.25 µg of L-thyroxine and 75 µg of cortisol every day. The animals were killed by decapitation 3–4 days after the operation.

IGF-II binding

The epididymal fat pads were immediately excised and minced. Isolated adipocytes were obtained by incubating the minced tissue in medium 199 with BSA (40 mg/ml) and collagenase (1 mg/ml) at 37 °C for 60 min. The cells were then filtered through a nylon mesh and washed four times in fresh medium. The preincubations were performed for 30 min at 37 °C, with a lipocrit of around 10 %, with or without the different agents indicated. Receptor cycling was then stopped by the addition of 2 mM-KCN 10 min prior to the binding assay. The cells were transferred to 24 °C, 0.1–0.25 ng of ¹²⁵I-IGF-II/ml was added and the incubations were carried out for 60 min when steady-state binding had been reached. The cell-associated radioactivity was determined in aliquots after separating cells and medium by centrifugation through silicone oil. Non-specific binding was estimated as the cell-associated radioactivity remaining in the presence of a large excess of unlabelled IGF-II (200 ng/ml) in the binding assay. This was subtracted from total binding to obtain specific binding.

GH binding

Binding of ¹²⁵I-labelled hGH to isolated adipocytes was measured essentially as described by Fagin *et al.* (1980) with the modifications previously reported (Edén *et al.*, 1982a). hGH was iodinated by the lactoperoxidase method (Thorell & Johansson, 1971) to a specific radioactivity of 80–100 µCi/µg. The labelled hormone was purified over a Sephadex G-75 column on the day of the binding experiments. After preincubation as described above followed by the addition of 2 mM-KCN, 1.5 ml portions of the cells and medium were transferred to 24 °C and mixed with 0.1 ml of medium containing approx. 3 ng of ¹²⁵I-hGH with or without 10 µg of unlabelled hGH. After 2 h, portions of the cells and medium were layered over silicone oil and centrifuged in microfuge tubes (Gliemann *et al.*, 1972). Cell-associated radioactivity was measured and specific binding was determined by subtracting non-specific binding, estimated as radioactivity in the presence of 10 µg of unlabelled hGH, from total cell-associated radioactivity.

IGF-II binding to solubilized receptors

In some experiments, cells preincubated as described above were solubilized essentially as reported by Marshall *et al.* (1985) but with some modifications. Portions of cells and medium were solubilized in 0.4 % Triton X-100 for 60 min at 4 °C in the presence of the protease inhibitors leupeptin (100 µM), pepstatin (70 µM), aprotinin (10 µg/ml) and phenylmethanesulphonyl fluoride (500 µM). The receptors were precipitated together with IgG by using poly(ethylene glycol). After repeated vortex-mixing, the samples were centrifuged at 11 000 *g* for 5 min and the pellets were washed carefully three times. After resuspending the pellets in medium 199 containing 4 % BSA and the protease inhibitors, binding of ¹²⁵I-IGF-II (0.2 ng/ml) in the presence or absence of 200 ng of unlabelled IGF-II/ml was performed at 4 °C. After

20 h, receptors were again precipitated with IgG using poly(ethylene glycol). After careful washing of the precipitate three times, the radioactivity was determined. Specific ¹²⁵I-IGF-II binding was obtained by subtracting the non-specific binding, determined as the radioactivity remaining in the presence of 200 ng of unlabelled IGF-II/ml.

Statistics

Statistical significance of differences was tested with Student's two-tailed *t* test for paired data.

RESULTS

Binding of ¹²⁵I-IGF-II to unstimulated cells from Hx rats was 1.0 ± 0.7 pg/10⁵ cells (mean $\pm$ S.E.M., *n* = 7) which was approx 2-fold higher than the binding to cells from sham-operated animals (average increases 110 ± 43 %, *n* = 4).

Effect of insulin and the interaction with cyclic AMP

Insulin markedly increased (10–15-fold) the number of binding sites for IGF-II in the sham-operated animals (results not shown). Insulin also increased IGF-II binding in cells from Hx rats (*P* < 0.01), but this effect was considerably smaller (approx. 3-fold increase) than that in the sham-operated animals (Fig. 1). The marked insulin sensitivity in Hx cells is shown by the fact that 5 µunits/ml exerted almost a full stimulatory effect (approx. 80 % of the maximal binding) (Fig. 1).

The non-metabolizable cyclic AMP analogue *N*⁶-mbcAMP markedly decreased IGF-II binding (~ 50 %) in both the absence and the presence of insulin. Isoprenaline significantly reduced the effect of insulin, whereas binding in the absence of insulin was essentially unchanged (Fig. 1). Both *N*⁶-mbcAMP and

Fig. 1. Effects of insulin and cyclic AMP on IGF-II binding to adipocytes from Hx rats

Cells were preincubated for 30 min in the presence or absence of insulin and *N*⁶-mbcAMP (4 mM) or isoprenaline (1 µM, Iso). After the addition of 2 mM-KCN, the cells were allowed to equilibrate with ¹²⁵I-IGF-II (0.10–0.25 ng/ml) for 60 min and specific binding was determined. ¹²⁵I-IGF-II binding to control cells (no additions) was 1.0 ± 0.7 pg/10⁵ cells and was in each experiment set to 100 %. Data are means $\pm$ S.E.M. of three to seven separate experiments. ****P* < 0.001, ***P* < 0.01, **P* < 0.05 for comparisons between the corresponding control group (basal or insulin alone) and the incubations with cyclic AMP and isoprenaline respectively.

isoprenaline more markedly decreased the effect of the submaximal insulin concentration (Fig. 1), indicating induction of a marked insulin resistance.

Effect of GH and the interaction with cyclic AMP and adenosine

The action of GH was only characterized in Hx cells, since its effect was too variable in the sham-operated animals (results not shown). GH elicited a consistent stimulatory effect in Hx cells (average increase $55 \pm 10\%$, $n = 7$, $P < 0.01$) (Fig. 2). However, the responsiveness to GH was markedly attenuated in the presence of N^6 -mbcAMP, whereas isoprenaline produced a less marked inhibition (Fig. 2).

ADA, which degrades the endogenous adenosine accumulating in the incubation medium, reduced unstimulated IGF-II binding as well as the responsiveness to GH to a similar extent as did the cyclic AMP analogue (Fig. 2). Addition of the non-metabolizable

adenosine analogue PIA completely restored IGF-II binding in both the absence and the presence of GH.

The dose-response relationship for the stimulatory effect of GH on IGF-II binding was also studied. The EC_{50} (concn. causing 50% of maximal effect) for GH alone varied (12 ± 5 ng/ml; $n = 6$), but both the cyclic AMP analogue (Fig. 3a) and isoprenaline (Fig. 3b) consistently and significantly increased the EC_{50} by 2–4 fold ($P < 0.02$).

Solubilized IGF-II receptors

125 I-IGF-II binding to receptors from solubilized cells from both sham-operated and Hx animals was measured. IGF-II binding, expressed per μ g of protein, did not differ significantly between sham-operated and Hx cells, and no differences were seen in cells which had been preincubated with GH or insulin. However, when the data were calculated per cell, binding was approx. 40% lower in Hx as compared with sham-operated rats due to the fact that adipocytes from Hx rats are smaller and contain less protein per cell (results not shown).

Fig. 2. GH-stimulated IGF-II binding to adipocytes from Hx rats

Cells were preincubated for 30 min with or without 1 μ g of hGH/ml and the indicated additions (1 unit of ADA/ml, 1 μ M-PIA, 4 mM- N^6 -mbcAMP or 1 μ M-isoprenaline). The experimental procedures were as in Fig. 1. Data are means $\pm$ S.E.M. of three to seven separate experiments. ** $P < 0.01$, * $P < 0.05$ for comparisons with the corresponding control group (basal or hGH alone) using paired t -tests.

Fig. 4. Effects of different agents on specific 125 I-hGH binding

Adipocytes from Hx rats were preincubated for 30 min in the presence or absence of isoprenaline (Iso, 1 μ M), N^6 -mbcAMP (4 mM), ADA (1 unit/ml) or PIA (1 μ M). 125 I-hGH binding to isolated adipocytes was measured as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three to six experiments.

Fig. 3. Effects of N^6 -mbcAMP (a) and isoprenaline (b) on the dose-response relationship for GH-stimulated IGF-II binding

For incubation conditions, see legend to Fig. 1. Cells were preincubated for 30 min with different concentrations of hGH in the absence (●) or presence (▲) of 4 mM- N^6 -mbcAMP (a) and in the absence (●) or presence (■) of 1 μ M-isoprenaline (b). Results are from one representative experiment of three and are expressed as percentage of maximal hGH effect (= 100%).

GH binding

The possibility that the attenuated sensitivity and responsiveness to GH of IGF-II binding in the presence of N^6 -mbcAMP, isoprenaline and ADA were due to an impaired ability of the hormone to bind to its receptor was also studied. However, as shown in Fig. 4, incubation of adipocytes from Hx rats with these agents did not significantly change GH binding.

DISCUSSION

This study shows for the first time that the insulin-like effect of GH on IGF-II receptor distribution is inhibited by cyclic AMP and β -adrenergic stimulation in a similar way as that of insulin itself. Our findings suggest that the insulin-like effect of GH shares a common pathway with insulin which occurs at the post-binding level.

Under the experimental conditions currently employed, where the cells are energy-depleted after preincubation with GH or insulin, the increased IGF-II binding is due to a rapid redistribution of the binding sites from an intracellular pool to the plasma membrane rather than to changed affinity (Wardzala *et al.*, 1984; Lönnroth *et al.*, 1987*a,b*). The present data show that IGF-II binding to the total receptor pool from solubilized cells was unchanged whether or not the cells had been preincubated with GH or insulin.

In contrast with its acute insulin-like action, chronic GH exposure seems to exert an opposite effect, since IGF-II binding to Hx (GH-deficient) cells in the absence of hormones was increased by approx. 2-fold when compared with cells from sham-operated animals. This 2-fold difference clearly underestimates the redistribution of the IGF-II receptors, since the total number of binding sites per cell, calculated from the binding to solubilized receptors, was decreased by approx. 40% in Hx animals. This may be due to a more general decrease in protein synthesis, since total cellular protein content was decreased to the same extent in these cells.

The effect of insulin on IGF-II binding was lower in Hx (GH-deficient) cells (3-fold increase in binding) as compared with cells from sham-operated animals (10-15-fold increase). A similar decrease in the insulin effect has been described in Hx cells for both insulin-stimulated glucose transport (Schoenle *et al.*, 1979*a,b*) and insulin-stimulated redistribution of the glucose transporters (U. Smith, B. Kahn & S. Cushman, unpublished work), suggesting a more general effect of GH on the subcellular distribution of integral membrane proteins. However, when the reduction in the total cellular number of IGF-II receptors (40%) as well as the subcellular redistribution of the receptors to the plasma membranes in unstimulated cells (approx. 2-fold) are considered, it becomes clear that insulin action *per se* is not impaired in Hx cells or by lack of GH. Taken together, these observations indicate that GH exerts a chronic regulatory effect, which is opposite to its acute insulin-like effect, on the subcellular distribution of the IGF-II receptors between the plasma membrane and the intracellular pool. This concept is supported by our findings that giving GH to Hx rats reduced the stimulatory effect of GH on IGF-II binding *in vitro* (Lönnroth *et al.*, 1987*a*).

The present data on the adipocytes from Hx cells underscore the similarities between the effects of β -adrenergic stimulation and cyclic AMP on insulin action and on the acute insulin-like effect of GH on IGF-II binding. Thus isoprenaline and N^6 -mbcAMP induce a clear rightward shift in the dose-response curves for both GH and insulin, suggesting an impairment in the signalling mechanisms. β -Adrenergic stimulation and the cyclic AMP analogue also produce a significant reduction in the responsiveness to both hormones. However, some differences

were seen between β -adrenergic stimulation with isoprenaline and the effect of N^6 -mbcAMP, which is not hydrolysed by the cyclic AMP phosphodiesterase (Beebe *et al.*, 1984). When added at a maximally effective concentration, N^6 -mbcAMP reduced IGF-II binding by approx 50% both in the basal, unstimulated state and in the presence of GH and insulin. Isoprenaline, on the other hand, did not affect basal unstimulated IGF-II binding but attenuated the effects of both insulin and GH. The reason for this discrepancy between the cyclic AMP analogue and isoprenaline might be that the latter, at least under some circumstances, can increase the endogenous release of adenosine from cells (Fredholm & Sollevi, 1981; Gidday *et al.*, 1988). Adenosine, in turn, exerts an insulin-like effect on both glucose transport and IGF-II binding (Lönnroth *et al.*, 1987*b,c*; Kuroda *et al.*, 1987). Additional support for this concept is provided by data showing that low concentrations of isoprenaline can stimulate glucose transport in the absence but not in the presence of ADA (Kashiwagi *et al.*, 1983).

Removal of adenosine with ADA decreased IGF-II binding to a similar extent as did the cyclic AMP analogue. The action of ADA was specifically due to adenosine removal, since concomitant addition of the non-metabolizable adenosine analogue PIA completely restored IGF-II binding to normal. Thus these findings suggest that adenosine, through its action on an inhibitory GTP-binding protein (G_i) not only influences insulin action but also facilitates the insulin-like effect of GH.

GH has been shown to exert its effects through an interaction with its specific receptor without cross-reacting with receptors for other peptide hormones (Fagin *et al.*, 1980; Edén *et al.*, 1982*b*). Neither β -adrenergic stimulation nor exposure to the cyclic AMP analogue induced any significant change in GH binding, even though they shifted the dose-response curve for GH to the right.

Recently, Gorin *et al.* (1988) reported that both β -adrenergic stimulation and cyclic AMP reduced GH binding to rat fat cells. However, in that study, the cells were exposed to the adrenergic agents for a longer time than was used in the present investigation. Furthermore, receptor internalization was not blocked before the binding assay. Still, when the time course for the effect of these agents on GH binding was analysed, it is clear that exposure to dibutyryl cyclic AMP for 30 min only slightly decreased binding (to approx. 80% of control). In the present study, GH binding was measured after stopping receptor internalization and recycling through energy depletion. This approach probably better reflects cell-surface GH binding under the defined preincubation conditions used. Our data, therefore, indicate that the resistance to GH occurs at a level distal to the GH-binding site. This observation, together with the many similarities in the effects exerted by β -adrenergic stimulation and the cyclic AMP analogue on GH and insulin action, suggests the existence of a common post-receptor pathway for these hormones. This is also supported by the similar mechanisms of action at the molecular level of both insulin and the insulin-like effect of GH with regard to the dephosphorylation of the hormone-sensitive lipase as a mechanism for their anti-lipolytic effect (Björgell *et al.*, 1984).

It is tempting to speculate that cyclic AMP interacts with an early post-binding phosphorylation event elicited by both GH and insulin, since β -adrenergic stimulation and cyclic AMP interfere with the tyrosine kinase activity of the insulin receptor (Stadtmauer & Rosen, 1986; Häring *et al.*, 1986). Recent data have also demonstrated an autophosphorylation of the GH-receptor at tyrosine sites in response to GH (Foster *et al.*, 1988). However, this finding is not compatible with the amino acid sequence of the cloned GH receptor, which does not include a typical tyrosine kinase sequence (Leung *et al.*, 1987; Wallis,

1987). Furthermore, there is no evidence that GH can directly stimulate the tyrosine kinase activity of the insulin receptor (D. Tornqvist, unpublished work). One possibility which would reconcile these observations is the existence of a subgroup of GH receptors carrying tyrosine kinase activity.

In conclusion, the present study demonstrates marked similarities between insulin and GH action, including counter-regulation by cyclic AMP. The data indicate a common post-receptor pathway for these hormones.

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