The acute GH action in rat adipocytes is associated with enhanced phosphorylation of a 46 kDa plasma membrane protein enriched by GH-Sepharose

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The acute anti-lipolytic effect of human growth hormone (hGH) in maximally noradrenaline-stimulated intact rat adipocytes was selectively associated with increased phosphorylation of a 46 kDa plasma membrane protein which was highly enriched by hGH-Sepharose chromatography. The same protein was also phosphorylated by an endogenous protein kinase in isolated plasma membranes, although then no hGH effect could be demonstrated. About 14% of the phosphate incorporated into the protein in isolated plasma membranes was found in tyrosine residues and the remainder in serine and threonine. The possible relation of the 46 kDa protein with the hGH plasma membrane receptor is discussed.

Somatotropin *Lipolysis* *Plasma membrane* *Phosphorylation*

1. INTRODUCTION

hGH possesses diabetogenic and, when given to hypophysectomised animals, insulin-like properties [1,2]. We have recently shown that in in vitro the acute anti-lipolytic (insulin-like) effect of hGH (which may also be seen with adipocytes from normal rats when no hGH has been present in the medium during the first 3 h after isolation) on maximally noradrenaline-stimulated isolated rat adipocytes is the result of a decreased phosphorylation of the hormone-sensitive lipase, as with insulin [3]. In the case of insulin, one generally held view is that the signal chain is initiated by the phosphorylation and thereby the activation of the insulin receptor β-subunit tyrosine kinase, with subsequent amplification of the signal through a cascade of intracellular protein phosphorylations and/or dephosphorylations. In view of the observed similarity between the mechanisms for the anti-lipolytic effect caused by the two hormones, we have speculated on the existence of a common intracellular signal chain starting from distinct plasma membrane receptors [4]. As a starting point to approach experimentally this question, we have examined the phosphorylation of adipocyte plasma membrane protein(s) associated with the anti-lipolytic effect of hGH.

Abbreviations: BSA, bovine serum albumin; hGH, human growth hormone; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride

2. MATERIALS AND METHODS

BSA fraction V (Sigma) was dialysed and defatted before use [5]. Hepes was bought from Schwartz-Mann. All concentrations are final concentrations. Fat cells were isolated from epididymal fat pads of 120–150 g Sprague-Dawley rats, age 31–36 days with collagenase (Worthington) in Hepes buffer [6]. After 2 h the cells were suspended at a final concentration of 5% (v/v) in fresh Krebs-Ringer buffer without phosphate. The metabolic integrity of the cells was ascertained regularly [7].

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26 ml of the cell suspension containing 5 mCi of [^{32}P]orthophosphate (Amersham, final concentration 50 μM) were preincubated for 50 min at pH 7.40 and 37°C in each of two pH-stat titrator vials. After 50 min, L-noradrenaline bitartrate (Sigma, final concentration 100 nM) was added to both samples and the fatty acid release measured by pH-stat titration [8]. At the peak of the fatty acid release (see fig.1) hGH (Crescormon, KabiVitrum, Sweden), 700 nM, was added to one of the samples. When the inhibition of the noradrenaline-induced lipolysis by hGH was maximal, the cells of both suspensions were quickly washed by centrifugation three times in ice-cold 20 mM Tris-HCl, pH 7.4, and 255 mM sucrose containing 1 mM ATP, 50 mM NaF, 1 mM EDTA and 10 μg/ml each of leupeptin, pepstatin and antipain (Peptide Institute). All the subsequent steps were carried out at 4°C.

The cells were homogenised in a Potter-Elvehjelm tissue grinder and the homogenates were then fractionated [9] to produce a plasma membrane fraction, a high density and a Golgi-enriched low density microsomal fraction. The remaining 400000 × g_{max} supernatant was designated the cytosolic fraction. The plasma membrane fraction was obtained from a Percoll gradient [9] by suspension of the initial 16000 × g_{max} pellet after it had been washed in homogenisation medium and pelleted once more at 16000 × g_{max}. After the Percoll had been removed [9], the plasma membrane fractions were solubilised in 3.9 ml of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 255 mM sucrose and 1% Triton X-100 by vigorous shaking for 1 h. The small remnant of insoluble matter was removed by centrifugation at 150000 × g_{max} for another hour.

The solubilised plasma membrane proteins were fractionated by affinity chromatography on hGH immobilised to CNBr-activated Sepharose 4B (Pharmacia), 1 ml bead volume, equilibrated with 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 255 mM sucrose and 0.1% Triton X-100 (column buffer). After the protein solutions had been added to the column it was washed with 4 ml of the column buffer. Finally, the column was eluted with column buffer made 3 M with respect to KI and 4 ml of the effluent was collected.

Both the 'column washings' and the 3 M KI eluates were concentrated to 0.1 ml using microconcentrators (Centricon 10, Amicon). Finally, the samples were precipitated and membrane lipids and the Triton X-100 were extracted [10] and the samples subjected to SDS-PAGE in 7.5% gels [11].

Other experiments were made with plasma membranes instead of intact cells. Epididymal cells were isolated and the cells were preincubated for 3 h in the absence of hormones to make them non-refractory to the anti-lipolytic action of hGH. Plasma membranes were prepared as described above and suspended in 20 mM Hepes, pH 7.4, 3 mM $MnCl_2$ and 0.1% BSA. Portions containing 270 μg of membrane protein were taken in plastic tubes and put on ice. Buffer (control) or hGH solution (700 nM) was added to a final volume of 375 μl. After 10 min, 125 μl of 60 μM ATP containing 10^8 dpm [γ-^{32}P]ATP was added to each tube. After another 10 min, 0.5 ml of 1 mM Na_3VO_4, 0.01 M $Na_2P_2O_7$, 100 mM NaF, 2 mM EDTA and 2 mM PMSF was added. The plasma membrane suspensions were pelleted by centrifugation and washed once with this buffer. The 46 kDa phosphoprotein (see below) was then isolated as described above.

After SDS-PAGE and staining with Coomassie blue G-250 the gels were either dried and autoradiographed or the protein in a gel slice corresponding to the 46 kDa phosphoprotein (see below) band electroeluted (ISCO model 1750 electrophoretic sample concentrator) and subjected to phosphoamino acid analysis [12]. BSA was used as standard for protein determination [13].

3. RESULTS AND DISCUSSION

The addition of hGH to the adipocytes caused rapid inhibition of the maximally noradrenaline-stimulated lipolysis (fig.1) (cf. [3]). In four experiments with separate cell batches hGH was added 8–11 min after noradrenaline, when the rate of lipolysis was approximately maximal. The adipocytes were harvested after another 10 min at the time point of maximal hGH effect (fig.1).

In each of the experiments 5% of the total adipocyte plasma membrane proteins bound to and could be eluted from the hGH-Sepharose with the 3 M KI-buffer (fig.2a). The most prominent of the adsorbed proteins with an M_r = 75000 enriched on the hGH-Sepharose column did not incorporate any measurable ^{32}P (fig.2b). However, one of the

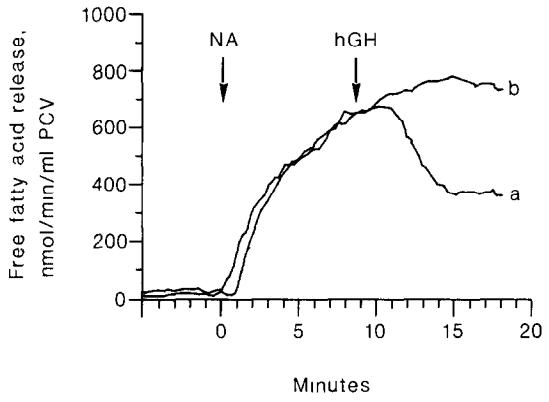

Fig.1. Recorder tracings obtained simultaneously from two pH-stat titrators. One titrator (a) contained a cell suspension to which was added noradrenaline (NA) and then hGH. The suspension in the other titrator (b) only received noradrenaline. The arrows indicate the time of addition of noradrenaline and hGH, respectively.

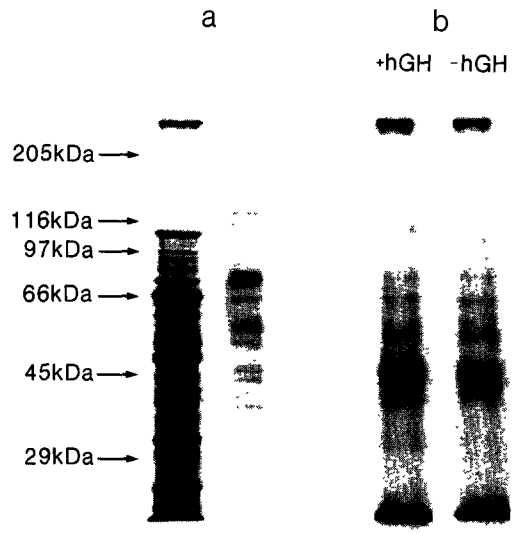

Fig.2. (a) The stained electropherograms of adipocyte plasma membranes (left) and the 3 M KI eluate from the hGH-Sepharose chromatography of solubilised adipocyte plasma membranes (right). (b) The autoradiograms after electrophoresis of the 3 M KI eluates from the hGH-Sepharose chromatography of the plasma membrane fractions from adipocytes incubated with hGH (+hGH) and from control adipocytes (−hGH). The autoradiograms were obtained with Kodak X-Omat RP film using Du Pont Cronex Lightning Plus Intensifying screen at −80°C for 1 week. The autoradiograms and the corresponding electropherograms were scanned with a Joyce Loebl Integrating Chromoscan 3 densitometer and the integrals from the former were corrected for differences in the contents of total protein in each lane using the total integral of stained protein bands of that lane. The molecular masses correspond to the location of the included protein standards (Sigma MW-SDS-200).

minor proteins (constituting <5% of the proteins adsorbed to hGH-Sepharose according to scanning densitometry), with an M_r = 46000, was found to be markedly phosphorylated in both control and hGH-treated cells (fig.2b). The extent of phosphorylation of this protein was selectively increased in cells incubated with hGH and in four experiments with different cell batches it exceeded that of the control cells by 32 ± 5% (mean ± SE, $p < 0.02$ with Student's t-test). Weak phosphorylation was also noticed in some of the other proteins which bound to the hGH-Sepharose but no reproducible effect of hGH on the extent of phosphorylation could be detected, nor was there any reproducible effect on those phosphorylated plasma membrane proteins which did not bind to the hGH-Sepharose (not shown). No ^{32}P-phosphorylated 46 kDa protein species could be detected in the Golgi-enriched low density microsomal or the cytosolic fractions. This makes it unlikely that the presence of the 46 kDa phosphoprotein in the plasma membrane fraction was due to contamination with these subcellular fractions.

In preliminary experiments, phosphoamino acid analysis of the 46 kDa phosphoprotein revealed mostly phosphoserine. There were also indications of some tyrosine phosphorylation but insufficient to allow quantitation. In order to examine this further we turned to studies with isolated plasma membranes incubated in vitro with [γ-^{32}P]ATP (cf. [14,15]). Also in the plasma membranes, the 46 kDa protein was phosphorylated but no measurable effect of hGH was found in spite of variations in the incubation conditions (not shown). In this case the phosphoamino acids could be analysed quantitatively and 14% of the ^{32}P (as estimated from scanning densitometry) was found on tyrosine (with and without hGH), with the remainder on serine and threonine (fig.3). It is possible that a cytosolic factor is required for hGH-dependent phosphorylation to occur.

The magnitude of the effect of hGH on the phosphorylation of the 46 kDa protein may be an

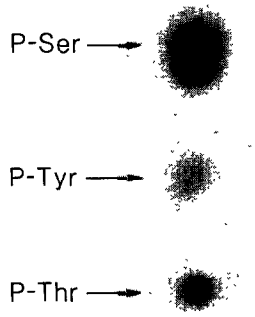

Fig.3. Phosphoamino acid composition of the 46 kDa protein band (see text) excised from the polyacrylamide gel after SDS-PAGE. The diagram represents the autoradiography after the electrophoretic separation of the acid hydrolysate of the 46 kDa band. Phosphoserine, -tyrosine and -threonine (P-Ser, P-Tyr and P-Thr, respectively) were added as carriers before electrophoresis and identified in the electropherogram after ninhydrin staining.

underestimate because of protein phosphatase catalysed dephosphorylation during the isolation procedure in spite of the use of phosphatase inhibitors. We have noticed that the phosphorylation of ATP citrate-lyase was increased three-fold by hGH-stimulation of adipocytes under the same experimental conditions when measured by direct fractionation of the total adipocyte proteins by SDS-PAGE (not shown). When determined in the cytosolic fraction, prepared as described above, only a 50% increase was found.

As to the identity of the 46 kDa phosphoprotein reported in this paper we can only speculate whether it has any relation to the hGH receptor. We have not been able to obtain reliable values of specific hormone binding to adipocyte plasma membrane protein due to the high background of non-specific binding after detergent solubilisation of the plasma membranes. Recently, it was shown that the hGH-binding receptor subunit which can be cross-linked to hGH in IM-9 lymphocytes does not become phosphorylated upon hGH-binding [16]. The corresponding cross-linked receptor-hormone complex of the rat adipocyte has an M_r = 134000 [17]. Naturally, it is tempting to speculate that, in analogy with the insulin receptor structure, this corresponds to a hormone binding α subunit and that the 46 kDa protein represents an autophosphorylated β subunit. A native receptor containing two of each such subunits would fit with the reported molecular mass of 300 kDa for the rabbit liver growth hormone receptor [18].

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