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Insulin induces a similar reduction in the concentrations of its own receptor and of an insulin-sensitive glycosyl-phosphatidylinositol in isolated rat hepatocytes

Juan Miguel Ruiz-Albusac, José Antonio Zueco, Esther Velázquez, José Félix Alvarez*, José María Mato* and Enrique Blázquez

Departamento de Bioquímica, Facultad de Medicina, Universidad Complutense, 28040-Madrid and **Departamento de Metabolismo, Nutrición y Hormonas, Fundación Jiménez Díaz and Instituto de Investigaciones Biomédicas del CSIC Reyes Católicos 2, 28040-Madrid, Spain*

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We have used isolated rat hepatocytes to study whether the insulin-induced reduction of its own receptors may modify the transduction of hormone signals by changes in the content of a glycosyl-phosphatidylinositol Both subsequent insulin binding and glycosyl-phosphatidylinositol concentrations markedly decreased as a function of time and insulin concentration during preincubation of hepatocytes with insulin The modifications observed in insulin binding were due to changes in receptor concentration These results show that insulin regulates both the number of its own receptors and glycosyl-phosphatidylinositol concentrations in target cells, which may be of interest in many pathophysiological situations

Insulin, Down-regulation, Insulin receptor, Glycosyl-phosphatidylinositol, Hepatocyte

1. INTRODUCTION

It is well known that insulin is required to activate anabolic processes in most mammalian tissues. However, the molecular events involved in the mechanism of insulin action are only partially understood, for lack of information on events occurring between the occupation of the insulin receptor and the biological effects of this hormone. Recently, insulin was shown to promote the hydrolysis of a glycosyl-phosphatidylinositol (glycosyl-PI) with release of its polar head-group [1–4]. This constitutes a link in the transduction of insulin receptor signals, mimicking the direct effects of insulin on the phosphorylation and dephosphorylation of a variety of target proteins [5]. There is evidence of an inverse relationship between the number of insulin receptors in target cells and the insulin concentrations in the extracellular space [6]. This phenomenon, commonly referred to as down-regularion, modulates the biological effects of the hormone in many physiological and pathological conditions [7,8]. In order to investigate whether the insulin-induced reduction of its own receptors modifies the transduction of hormone signals by changes in the content of the glycosyl-PI, we studied both components in isolated rat hepatocytes preincubated in the absence or presence of different insulin concentrations.

Correspondence address. J M Ruiz-Albusac, Departamento de Bioquímica, Facultad de Medicina, Universidad Complutense, 28040-Madrid, Spain

2. MATERIALS AND METHODS

Isolated hepatocytes were obtained from male Wistar rats (200–250 g) as previously described [9] Cell viability was improved by applying the cell suspensions to a Percoll gradient Isolated hepatocytes were preincubated at 37°C with a selective medium (Dulbecco and Voght's modification of Eagle's medium), deficient in arginine which suppresses the viability of nonparenchymal liver cells [10], supplemented with 2% bovine serum albumin (BSA), penicillin and streptomycin (225 U/ml and 45 μg/ml, respectively) for 0–6 h in the absence or presence of different insulin concentrations (10^{-9}–10^{-6} M) After preincubation, the cell suspensions were centrifuged for 2 min at 60 × *g* and immediately resuspended in 7 ml of Krebs-Hepes (25 mM) buffer, pH 7.4, containing 1% BSA The cell suspensions were washed, recentrifuged and incubated with another 5 ml of buffer at 37°C for 30 min This procedure was expected to be sufficient to dissociate all the insulin molecules bound to receptors during the preincubation period Alternatively, insulin was dissociated from its cell surface receptors by treating hepatocytes with Krebs-Hepes (25 mM) buffer, pH 4, for 5 min at 4°C. Cell suspensions were then centrifuged at 60 × *g* for 2 min and resuspended in Krebs-Hepes (25 mM) buffer, pH 7.4 Similar results were obtained with both insulin dissociation procedures

For insulin binding studies, isolated hepatocytes (10^6 cells/ml) were incubated with Krebs-Hepes (25 mM) buffer, pH 7 4, supplemented with 1% BSA, bacitracin (0.5 mg/ml) and [^{125}I]-insulin (5×10^{-11} M) at 30°C for 1 h The differences between cell-bound radioactivity in the presence and absence of an excess of unlabelled hormone (1 μM) was considered to represent bidning of the labelled hormone to specific binding sites Cell-bound hormone was separated from the free hormone by microfiltration Insulin association and dissociation from isolated hepatocytes and insulin inactivation studies were carried out as described in [11] Mono[^{125}I]-insulin with specific activities of 240–300 μCi/μg, was obtained by the procedure of Roth [12]

Glycosyl-PI was isolated from the isolated hepatocytes (10^6 cells) immediately after the preincubation period by a modification of the

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method described in [13] Cells were extracted with 30 ml chloroform/methanol (1 2)/0 05 N HCl After generation of two phases by the addition of 10 ml chloroform and 10 ml 0 1 M KCl, 4 g silica gel G (activated at 110°C for 1 h) and 40 ml chloroform were added to the organic phase The silica gel was washed twice with 40 ml chloroform/methanol/HCl (300 . 50 3) to remove common phospholipids, and the glycosyl-PI eluted with 40 ml methanol After drying under a stream of nitrogen, each sample containing glycosyl-PI was sonicated for 10 min in 0 3 ml of 5 mM sodium phosphate buffer, pH 8 0, and then reacted with 5–7 μCi [1-^{14}C]isethionyl acetimidate (Amersham, England, 50–60 mCi/mmol) The reaction was terminated by the addition of 3 ml chloroform/methanol (1 2)/0 05 N HCl and the amidinated ^{14}C-labelled glycosyl-PI purified by sequential TLC The first TLC was developed twice in chloroform/acetone/methanol/acetic acid/water (10 4 2 2 1) and the second TLC in chloroform/methanol/NH_4OH/water (90 90 7 20) After the last chromatography, 1-cm fractions were scraped off and the radioactivity associated with the glycosyl-PI determined [13]

The results are expressed as mean ± S E M For statistical comparisons, Student's *t*-test or one-way analyses of variance (ANOVA) were used when appropriate The mathematical approach used to calculate the affinity constants and the number of binding sites in the Scatchard plots was a curve-fitting program based on a model that analyses multiple independent classes of sites [14]

3. RESULTS AND DISCUSSION

Liver cell suspensions, which were 90–95% viable as judged by their ability to exclude 0.2% Trypan blue, bound insulin and glucagon specifically and responded to glucagon by an increase in the production of cyclic-AMP and in the rate of gluconeogenesis from alanine. Insulin binding to the isolated hepatocytes decreased significantly as a function of the time of preincubation with insulin and of the concentration of this hormone (figs.1, 2, and table 1). These findings seem to be specific for insulin binding sites, since preincubation of hepatocytes in the presence or absence of insulin did not modify glucagon binding to these cells [11]. The modifications observed in insulin binding were due to changes in receptor concentrations (fig.1 and table 1). Studies of the kinetic properties of the insulin receptors in the different experimental groups revealed that association and dissociation rates were indistinguishable (data not shown). Thus, when maximal binding of [^{125}I]insulin to isolated hepatocytes preincubated in the presence or absence of unlabelled insulin was normalized, the time courses of association were similar. Maximum binding of insulin occurred after 60 min of incubation at 30°C. Dissocation of [^{125}I]insulin from isolated hepatocytes was rapidly and significantly accelerated by addition of unlabelled insulin, with a similar pattern in all groups of cells studied. In addition, insulin inactivation, expressed as percent/10^6 cells/h at 30°C, was similar in all experimental groups (approximately 2.8%).

As previously reported [13], the free amino group of the residue of glucosamine in the purified glycosyl-PI can be amidinated with the imidoester [1-^{14}C]isethionyl acetimidate (IAI). Although other amino-phospholipids, such as phosphatidylethanolamine and phosphatidylserine, can also react with IAI [15], the procedure used permits the isolation of ^{14}C-labelled amidinated glycosyl-PI from other amidinated lipids [13]. That the isolated product was derived from the insulin-sensitive glycosyl-PI was shown by the fact that, as previously described [3], the ^{14}C-labelled amidinated glycosyl-PI was insulin-sensitive and could be hydrolys-

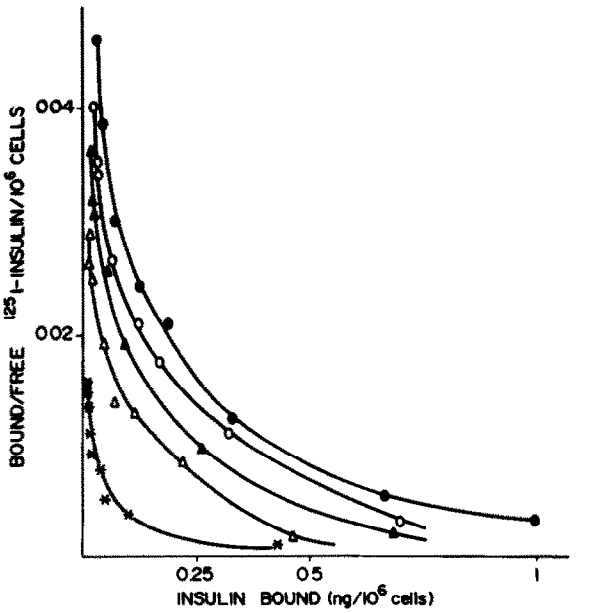

Fig 1 Scatchard analysis of insulin binding to isolated hepatocytes of adult rats previously preincubated at 37°C for 1 h in the absence (●) or presence of 10^{-9} (○), 10^{-8} (▲), 10^{-7} (△), 10^{-6} (★) M insulin Insulin binding was determined as described in section 2, with (0 15–2000 ng/ml) or without unlabeled insulin at 30°C Points are the means of at least 3 independent experiments

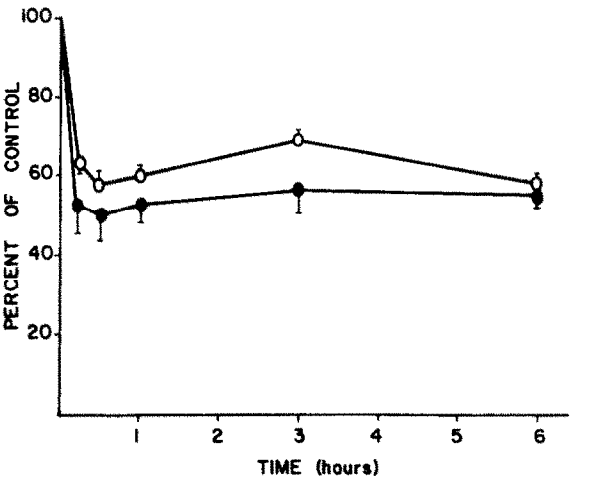

Fig 2 Effect of preincubation with 10^{-6} M insulin on insulin binding (○) and on glycosyl-phosphatidylinositol content (●) in isolated hepatocytes of adult rats Results are expressed as percentage change from cells preincubated in the absence of insulin (control) Means ± S E M (n = 3-6)

Table 1

Concentration of high- and low-affinity insulin binding sites and their affinities in adult rat isolated hepatocytes previously preincubated at 37°C for 1 h in the absence (control) or presence of insulin

	Control	Hepatocytes preincubated with different insulin concentrations (M)			
		10^{-9}	10^{-8}	10^{-7}	10^{-6}
Affinity constants ($\times 10^9$ M)					
High affinity (K_{d_1})	1 53 ± 0 12	1 50 ± 0 09	1 41 ± 0 15	1 83 ± 0 24	1 89 ± 0 09
Low affinity (K_{d_2})	23 10 ± 1 28	25 90 ± 2 78	28 80 ± 3 78	43 80 ± 7 10	32 00 ± 0 47
Binding sites (insulin molecules/cell)					
High affinity/low capacity (B_1)	35 300 ± 785	28 600 ± 629***	21 600 ± 1669***	16 900 ± 2090***	12 200 ± 508***
Low affinity/high capacity (B_2)	93 400 ± 1617	87 600 ± 2396	81 000 ± 3152**	66 500 ± 4030***	51 600 ± 404***
Total number (B_{max})	128 700 ± 1801	116 200 ± 2477**	102 600 ± 3568***	83 400 ± 4538***	63 800 ± 652***

Insulin binding was determined according to the procedure described in the legend of fig.1. Mean ± SE One-way analyses of variance (ANOVA) were used The mathematical approach used to calculate the affinity constants and the number of binding sites in the Scatchard plot was a curve-fitting program based on a model that analyses multiple independent classes of sites
*$P<0$ 05, **$P<0$ 025, ***$P<0$ 005 vs control

ed by a PI-specific phospholipase C from *B. cereus* (data not shown). Furthermore, in cells labelled with [^{3}H]-glucosamine and then allowed to react with IAI, the ^{3}H-labelled glycosyl-PI copurified with the ^{14}C-labelled amidinated lipid (data not shown). Constant exposure of isolated hepatocytes with insulin resulted in a proportionate reduction of both insulin receptor and glycosyl-PI concentrations (figs.1, 2, and tables 1, 2). The magnitude of the diminution of GPI levels and insulin receptor number were dependent on the concentrations of insulin, with a correlation coefficient of $r=0.72$ and $r=0.77$, respectively. Reduction of insulin binding and of glycosyl-PI occur within the first hour of preincubation with insulin (fig.2) and then remain decreased for up to 6 h of preincubation.

A correlation between the levels of glycosyl-PI and the induction of insulin receptors has also been observed in T lymphocytes [16]. Similarly, in CHO cells bearing normal human insulin receptors, the levels of glycosyl-PI are 3-fold higher than in the parental cells and in cells bearing a mutant receptor that lacks tyrosine kinase activity [17].

Glycosyl-PI molecules are considered as precursors to signal transduction elements since treatment of isolated hepatocytes with physiological concentrations of insulin initiated the rapid loss and resynthesis of glycosyl-PI and the appearance of its polar head-group through the action of a PI-specific phospholipase C [2,3,16]. This polar head group has been reported to mimic insulin action, modulating cyclic AMP phosphodiesterase, pyruvate dehydrogenase and adenylate cyclase in cell extracts [18], through its effects on pyruvate kinase and glycogen phosphorylase in rat hepatocytes [19] and in its ability to copy the antilipolytic [20] and lipogenic [21] effects of insulin and the effects of this hormone on protein phosphorylation/dephosphorylation [5,22] and on phospholipid methyltransferase [23].

An inverse correlation between extracellular insulin concentrations and insulin binding to target cells has been observed in man and in experimental animals. In insulin-sensitive states, either genetic [7] or following adrenalectomy and hypophysectomy [24–26], there is hypoinsulinemia and an increased number of insulin receptors. Conversely, in insulin-resistant states, such as those in obesity and glucocorticoid excess [7,27–29] there is hyperinsulinemia and a decreased insulin binding to target cells. Our results indicate that insulin itself directly regulates not only the number of its receptors (fig.1) but also the concentration of glycosyl-PI (fig.2) in target cells. This may be of interest because for other hormones a reduction in the number of binding sites does not necessarily lead to a decrease in their biological activity [29].

If, as has been proposed, the phospho-oligosaccharide of this glycosyl-PI mediates some of the actions of insulin, the regulation by insulin of glycosyl-PI concentrations may be a mechanism of controlling the biological actions of the hormone. The study of this insulin-sensitive glycosyl-PI may open new insights in the transduction of signals in insulin-resistant states, such as in obesity and non-insulin-dependent diabetes, where

Table 2

Glycosyl-PI content in isolated hepatocytes previously preincubated for 1 h at 37°C in the absence (control) or presence of insulin

Insulin concentration (M)	Glycosyl-PI content (^{14}C cpm/μmol phospholipid)
0	6819 ± 318
10^{-9}	6588 ± 365
10^{-8}	5434 ± 390*
10^{-7}	4874 ± 254**
10^{-6}	4332 ± 127**

Results are the means ± S E of 3–6 independent experiments
*$P<0$ 01, **$P<0$ 005 vs control

a decreased biological action of the hormone is accompanied by hyperinsulinemia and a decreased number of insulin receptors [28]. In addition, in the insulin resistance of uremia, the numbers, affinity and structure of the insulin receptor and its tyrosine-specific kinase activity are normal in isolated hepatocytes, but these cells may fail to generate the chemical mediator of insulin action, as assessed by the inability of insulin to stimulate pyruvate dehydrogenase activity [30].

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