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Effect of glucagon on insulin receptor phosphorylation in intact liver cells

Tarik Issad*, Steven W. Young, Jeremy M. Tavaré and Richard M. Denton

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol, BS8 1TD, UK

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Evidence is presented that incubation of rat liver cells with glucagon leads to an increase in the phosphorylation of specific serine residues within insulin receptors, particularly in the presence of insulin. However, no changes in either the tyrosine phosphorylation of the receptors or the tyrosine kinase activity towards a synthetic peptide substrate was detected.

Insulin receptor; Liver; Glucagon; Insulin; Phosphorylation

1. INTRODUCTION

One of the first events following insulin binding to the α-subunit of the receptor is the increased autophosphorylation of the β-subunit on several tyrosine residues [1], which in turn stimulates the tyrosine kinase activity of the receptor towards other substrates. It is widely accepted that the receptor tyrosine kinase activity plays an important role in the transmission of the effects of insulin to intracellular systems (for review, see [2]). On the other hand, the phosphorylation of the insulin receptor on serine or threonine residues may have an inhibitory effect on the kinase activity of the receptors. Evidence for such a mechanism has been obtained for phorbol esters apparently acting through protein kinase C [3,4]. There is also some indication that agents acting through cyclic AMP may have an inhibitory effect on the activity of the insulin receptor although the evidence is in part contradictory and certainly incomplete. It has been reported that cyclic AMP-dependent protein kinase can phosphorylate the insulin receptor in vitro, with a small concomitant inhibition of the tyrosine kinase activity of the receptor [5], but others have failed to find any effect of cAMP-dependent protein kinase [6] or ascribed the inhibition of the receptor tyrosine kinase to a mechanism which is independent of receptor phosphorylation [7]. In intact cells, there is evidence that increasing intracellular cyclic AMP concentrations may result in a decrease in the tyrosine kinase activity of the insulin receptors [8–10]. However, in only one study [8] was the increase in cyclic AMP shown to be associated with an increase in the phosphoserine content of the receptor. The effect of increasing cell cyclic AMP concentrations on the phosphorylation of individual sites within the insulin receptor has not been examined previously.

Using a sensitive two-dimensional phosphopeptide mapping technique [11], we recently developed a procedure allowing the study of individual phosphorylation sites of insulin receptors in intact freshly isolated liver cells ([12] and Fig. 1). Some marked differences were apparent between the pattern of phosphorylation sites in the fresh liver cells and those observed in a range of cultured cells including hepatoma cells [13,14] expressing high levels of the receptor. In particular, in liver cells: (1) there was no phosphorylation of the tyrosines close to the carboxyl terminus (1328 and 1334) although insulin caused a large increase in the phosphorylation in the three tyrosines in the kinase domain (1158, 1162 and 1163); (2) the phosphorylation of the threonine (1348) near to the carboxyl terminus was low in the presence and absence of insulin; (3) there was extensive phosphorylation of serines within the receptor in cells incubated under basal conditions and only modest changes in serine phosphorylation with insulin [12].

In the present study, we have investigated the effects of increasing the concentration of cyclic AMP by glucagon on the phosphorylation of individual sites within the insulin receptors of intact fresh liver cells.

2. MATERIALS AND METHODS

2.2. *Phosphorylation of insulin receptors within intact liver cells*

This was performed as described in [15]. Hepatocytes were distributed in four incubation vials (25 million/vial) and preincubated for 50 min in 2 ml of Krebs–Hensleit buffer containing $^{32}P_i$ (0.2 mM, 1.5 mCi/ml), then the cells were incubated for 15 min in the absence and presence of glucagon (300 nM, Sigma, Poole, England); where indicated insulin (200 nM) was added for the last 10 min of the incubation. The cells were then rapidly extracted at 2–4°C in ice-cold HEPES buffer (100 mM, pH 7.6) containing 2% Triton X-100, 40 mM EDTA, 20 mM NaF, 60 mM sodium pyrophosphate, 4 mM benzamidine, 2

Present address: Centre de Recherche sur l'Endocrinologie Moléculaire et le Développement (C.N.R.S.), 9 rue J. Hetzel, 92190 Meudon-Bellevue, France.

Correspondence address: R.M. Denton, Dept. Biochemistry, School of Medical Sciences, University of Bristol, Bristol, BS8 1TD, UK.

mM Na_3VO_4, 2 mM phenylmethylsulphonyl fluoride and 2 μg/ml each of aprotinin, pepstatin, antipain and leupeptin.

2.2. *Purification and two-dimensional phosphopeptide mapping of the insulin receptors*

The purification and mapping procedures have been fully detailed in [11,12]. Fig. 1 provides a key to the various phosphopeptides derived from the insulin receptor. The phosphopeptides were detected by radioautography; densitometric scanning of the radioautographs was carried out using a Chromoscan 3 (Joyce Loebl Ltd., UK) linked to a Hewlett–Packard series 300 computer.

2.3. *Measurement of the exogenous kinase activity of receptors from liver cells incubated in absence or presence of glucagon and insulin*

Liver cells were prepared, incubated, extracted, and the receptors partially purified by wheat-germ lectin Sepharose chromatography as for ^{32}P labelling of the β-subunit, except that radioactive phosphate was omitted. A sample of partially purified receptor was removed and separated by SDS-PAGE for quantification of the amount of insulin receptor by Western blotting using chemiluminescent detection [15,16]. Exogenous kinase activity of the partially purified receptors was measured using a synthetic peptide RRDIFETDYFRK (designated as the FYF peptide) corresponding to the kinase domain of the human insulin receptor in which tyrosines 1158 and 1163 have been replaced by phenylalanine (kindly provided by Dr. L. Ellis, University of Texas Southwestern Medical Center, Dallas). Equivalent amounts of partially purified insulin receptors were pre-incubated for 5 min at 30°C in a final volume of 50 μl containing 25 mM HEPES, 1.5 mM EGTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, 12 mM $MgCl_2$ and 0.46 mM of FYF peptide. The reaction was then started by adding 2 μCi of [γ-^{32}P]ATP (final concentration 100 μM). The reaction was terminated by spotting a sample of the incubation mixture onto phosphocellulose P81 paper squares (Whatman Labsales Ltd., Kent, UK). The papers were immersed in 0.5 litre of 150 mM H_3PO_4 and washed 3 times for 15 min with fresh 150 mM H_3PO_4 and once in ethanol, dried and counted. Background radioactivity was determined for each incubation condition by performing an identical reaction in the absence of substrate peptide.

3. RESULTS

The cells were incubated in the absence or presence of glucagon for 15 min and when appropriate insulin was added for the final 10 min. High concentrations of hormones were employed to ensure that maximal effects might be achieved. Fig. 2 shows a typical example of two-dimensional phosphopeptide maps of insulin receptors obtained under these conditions. Changes in the amounts of the various phosphopeptides in this experiment plus two others carried out under the same conditions are shown in Table I. The table gives the effects of glucagon both in the absence and presence of insulin. In addition, the proportion of overall phosphorylation that can be ascribed to individual peptides are also given in order to correct for any differences in recovery that might occur during the preparation of the receptor and its phosphopeptides. The overall incorporation of radioactivity into the β-subunit of the insulin receptor was 1982±470 (mean±SEM of 3 observations in the absence of hormones). This was increased to 4264±859 in the presence of insulin. Glucagon did not have a significant effect on the overall incorporation of radioactivity in either the absence or presence of insulin (Table I).

The pattern of peptide phosphorylation in the absence of hormones and with insulin alone were in excellent agreement with those obtained previously under essentially the same conditions [15]. Glucagon had no detectable effect on the phosphorylation of tyrosines within the insulin receptor both in the absence and presence of insulin. It should be noted that the level of

Table I

Effect of glucagon on the phosphorylation of the insulin receptor in intact liver cells incubated in the presence and absence of insulin. Results are taken from the experiment shown in Fig. 2 together with two further independent experiments carried out in the same way. Relative phosphorylation was determined by scanning of the radioautographs and is expressed as means ±SEM of three observations. A key to the phosphopeptides is given in Fig. 1. *$P<0.05$ for the effect of glucagon versus appropriate control.

	Effect of glucagon (%)		Phosphorylation (as % of overall phosphorylation)			
Peptide	In absence of insulin	In presence of insulin	Control	Glucagon	Insulin	Insulin + glucagon
Kinase domain						
Monophos. forms (C1)	–	74±7.8	11±5.0	8±1.9	14±1.4	11±1.8
Bisphos. forms (B2 + B3)	–	94±30	5±2.6	3±1.2	21±0.5	17±1.9
Trisphos. forms (A1 + A2)	–	100±20	4±2.0	6±2.4	45±2.1	43±3.3
All phosphotyrosine containing		94±20	20±8.6	17±4.0	81±1.7	71±2.6*
Phosphoserine containing						
X1	124±28	274±42*	29±7.9	25±2.2	5±0.5	13±2.2*
X2	71±26	121±43	14±1.0	7±1.2*	4±0.7	3±0.5
X5	338±84	408±243	12±1.3	30±5.6*	3±1.1	7±1.7
All non-phosphotyrosine containing (X1-X5)	132±32	179±61	80±8.6	83±4.0	19±1.7	29±2.6*
Overall	134±41	108±26	100	100	100	100

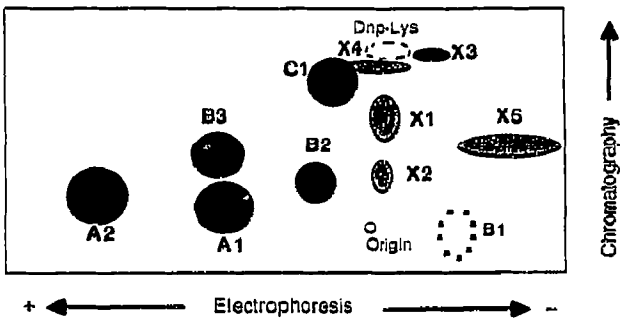

Fig. 1. Key to the phosphopeptides derived from rat insulin receptors phosphorylated in intact liver cells. Taken from [11 and 12]. For ease of comparison, we have numbered amino acids in the insulin receptor according to the human sequence described in [20]. The amino acid sequence of the β-subunit of the rat receptor is very similar to the human sequence but there is an insert of two amino acids in the α-chain [21]. Autophosphorylation of the tyrosines 1158, 1162 and 1163 in the kinase domain can give rise to a family of five phosphopeptides (general sequence Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys) which are mono-(C1), bis-(B2 and B3) or tris-(A1 and A2) phosphorylated and cleaved by trypsin at Arg-1155 and either Arg-1164 (C1, B3 and A2) or Lys-1165 (B2 and A1). [11]. The other phosphopeptides are phosphoserine containing peptides with the exception of X3, which is a phosphothreonine containing peptide VLTLPR ([12] and unpublished observations). In intact liver cells, the two tyrosines from the carboxylterminus (1328 and 1334) are not phosphorylated and hence phosphopeptide B1 is absent [12].

phosphorylation of tyrosines in the kinase domain in the absence of insulin was too low both with and without glucagon for reliable percent changes in phosphorylation to be calculated and hence these are not included in Table I. On the other hand, glucagon did cause increases in the phosphorylation of two phosphoserine containing peptides X1 and X5. Incorporation into X5 increased both in the absence and presence of insulin. However, the increases were variable in extent and only reached statistical significance using Students's *t*-test when the results are expressed as percent of overall phosphorylation. X5 may not represent a single polypeptide as it does not run as a discrete spot in the two-dimensional maps. Both X1 and X5 were found to contain only phosphoserine when derived from receptors of cells exposed to glucagon (X1) or insulin plus glucagon (X1 & X5). Glucagon stimulation of phosphorylation of X1 appeared only to increase in the presence of insulin when there was a two- to three-fold enhancement. Overall, in the presence of insulin the effect of glucagon was to more than double the ratio of phosphorylation of the two major phosphoserine containing peptides (X1 and X5) to that of the major phosphotyrosine containing peptides (A1,A2,B2,B3 and C1) from 0.13±0.02 to 0.27±0.03 (3 observations). Precise stoichiometries cannot be calculated from the data given in Table I; however, it can be concluded that in the presence of insulin and glucagon, the number of receptors phosphorylated on all three tyrosines in the kinase domain (i.e. yielding peptides A1 and A2) is approximately the same as the number phosphorylated on peptide X1 (assuming this peptide only contains a single phosphoserine).

In order to assess the extent to which the changes in phosphorylation of X1 and X5 with glucagon may be able to influence the tyrosine kinase activity of the insulin receptor, receptors were partially purified by wheat-

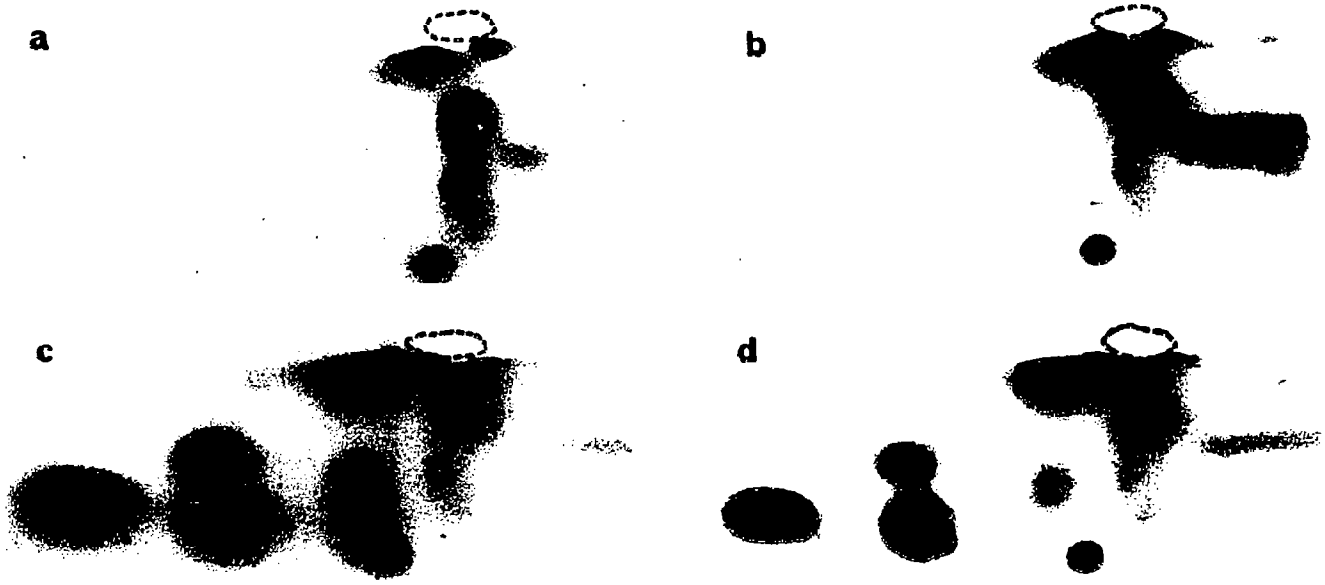

Fig. 2. Two-dimensional maps of the phosphopeptides from liver cells. After preincubation, liver cells were incubated 15 min either in the absence (panel A and C) or presence (panel B and D) of glucagon (300 nM). In panels C and D, insulin (200 nM) was added for the last 10 min of incubation. The position of an internal marker, DNP-lysine (dotted oval) is shown.

germ lectin affinity chromatography from cells treated with hormones (under conditions used for peptide mapping) and the tyrosine kinase activity towards the FYF peptide determined. As can be seen from the results given in Fig. 3, kinase activity was increased more than ten-fold in the presence of insulin as expected from the increase in tyrosine phosphorylation within the kinase domain. However, there was no evidence of any changes following exposure of liver cells to glucagon.

4. DISCUSSION

This study represents the first analysis of the changes in the phosphorylation of the insulin receptor in freshly isolated cells treated with a hormone which increases cyclic AMP. Evidence has been obtained that exposure of hepatocytes to glucagon results in modest increases in the phosphorylation of the insulin receptor on serines within two peptides designated X1 and X5. In the case of X1, the increase was only evident in the presence of insulin and it is possible that insulin binding or the subsequent increase in tyrosine phosphorylation is a prerequisite before increased serine phosphorylation can occur within peptide X1. Further studies are required to establish the location of phosphopeptides X1 and X5 within the β-subunit of the insulin receptor and the protein serine kinases involved but cAMP-dependent protein kinase is unlikely to directly phosphorylate the insulin receptor [6,7]. Phosphopeptides X1 and X5 are clearly distinct from the tryptic peptide containing serines 1305 and 1306 which has been reported to be a major site of phosphorylation by the protein serine kinase which copurifies with the insulin receptor [17]. This phosphopeptide migrates further towards the cathode than X5 [16], and we barely detected any phosphorylation of this peptide within intact cells exposed to insulin [12,14,16 and this study], and in any case, the major phosphoserine containing peptides are still evident in mutated human insulin receptors lacking 69 amino acids from the carboxyl terminus of the β-subunit and transfected into CHO cells [18]. It is possible that the effects of glucagon may involve changes in the activity of phosphoserine protein phosphatase activity, for example through changes in the phosphorylation of inhibitor 1.

Despite the increase in phosphoserine content promoted by glucagon, we were unable to detect any changes in the extent of phosphorylation of insulin receptor tyrosines 1158, 1162 and 1163 or on exogenous tyrosine kinase activity subsequently assayed using a synthetic peptide substrate. These results contrast with those of Stadtmauer and Rosen [8] who obtained evidence that treatment of IM9 lymphoblasts with forskolin enhanced the basal phosphorylation of insulin receptors apparently mainly on serine residues and decreases the stimulatory effect of insulin on tyrosine phosphorylation of the receptors, although the individual phosphorylation

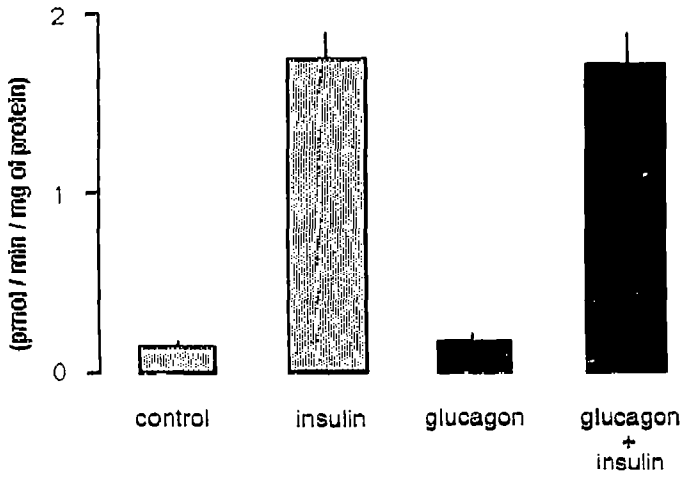

Fig. 3. Effect of glucagon treatment on the exogenous tyrosine kinase activity of insulin receptors. Liver cells were incubated as in Fig. 2, extracted and the receptors partially purified by chromatography on wheat-germ lectin Sepharose and assayed for tyrosine kinase activity towards peptide FYF (see section 2). Equivalent amounts of receptors were used in each incubation. Results are expressed in pmol of phosphate incorporated into the peptide/min/mg of proteins and represent the mean ±SEM of three separate experiments.

sites involves were not identified. It is possible that forskolin treatment of IM9 cells alters the phosphorylation of residues in the receptor not phosphorylated in the intact liver cell such as the two tyrosines near to the carboxyl terminus or that the high level of serine phosphorylation of the liver receptor is already inhibitory under basal conditions. Certainly, IM9 lymphoblasts are hardly typical insulin-sensitive cells and thus the effects of increasing cell cyclic AMP may not be representative of those occurring in the cells of insulin-sensitive target tissues. Although the results of the present study suggest that increases in the serine phosphorylation observed with glucagon do not affect the tyrosine kinase activity of the insulin receptor, it is still possible that the increases alter some other important property of the receptor such as its ability to interact with other intracellular proteins that may be involved in insulin signalling such as the 185K phosphotyrosine containing protein [19].

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