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Anti-Insulin Receptor Antibodies Mimic the Effects of Insulin on the Activities of Pyruvate Dehydrogenase and AcetylCoA Carboxylase and on Specific Protein Phosphorylation in Rat Epididymal Fat Cells

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Summary. Previous studies have shown that autoantibodies against insulin receptors found in certain patients with severe insulin resistance stimulate glucose transport and metabolism in fat cell and muscle preparations. The present studies show that preincubation of rat epididymal adipose tissue with 1 : 1000 dilution of one such serum results in a two fold increase in the initial activities of pyruvate dehydrogenase and acetylCoA carboxylase. These increases are similar to the maximum effects of insulin. Incubation of isolated fat cells with the serum at the same concentration also resulted in the increased phosphorylation of three intracellular proteins with subunit molecular weights of 130,000, 35,000 and 22,000 to the same extent as observed with insulin. These findings lend further support to the view that the short term effects of insulin do not involve the entry of the insulin molecule (or part thereof) into cells of target tissues.

Key words: Anti-insulin receptor antibodies, insulin effects, pyruvate dehydrogenase, acetylCoA carboxylase, specific protein phosphorylation, protein inhibitor (I_1) of the general phosphoprotein phosphatase.

Many important questions about the mechanism whereby insulin, after first binding to specific receptors on the outside of cells, causes changes in the activities of a range of key enzymes within cells remain unanswered [1]. One such question is whether the action of insulin requires that the insulin molecule (or part thereof) enters the cells of the target tissue [2–4].

Recent studies of Kahn and his colleagues have identified a group of patients characterized by severe insulin resistance and acanthosis nigricans who appear to have high concentrations of circulating antibodies to the insulin receptor present in the plasma membranes of muscle, adipose tissue and liver cells [5–8]. These antibodies have been found to stimulate glucose transport and metabolism in fat and muscle preparations to a similar extent to insulin [6, 8]. It seems reasonable to assume that if these immunoglobulins bring about changes in the activities of intracellular enzymes similar to those observed with insulin then it is unlikely that insulin's effects on those enzymes involves internalization of the whole or part of the insulin molecule [4]. Indeed, very recent studies by Lawrence and others have shown that the antibodies do bring about changes in the activities of glycogen synthase and phosphorylase in fat cells very similar to those observed with insulin [4]. However, in apparent contrast, the antibodies from the same patient did not appear to stimulate glycogen synthesis in the mouse soleus preparation to the same extent as insulin [8].

In the present study, we have explored the effects of the same serum on other intracellular processes in rat epididymal adipose tissue and fat cells which are affected by insulin. These include the activities of both pyruvate dehydrogenase and acetylCoA carboxylase, which are increased by about 2 fold within a few minutes of exposure of the tissue to insulin [9–12] and the phosphorylation of specific proteins within fat cells. This can be studied by incubating the cells with $^{32}P_i$ followed by extraction and the separation of the labelled phosphoproteins by SDS-polyacrylamide gel electrophoresis [13–15]. With this technique we have been able to demonstrate directly that the activation of pyruvate dehydrogenase in the presence of insulin is due to dephosphorylation of the enzyme [12, 15].

Phosphorylation of acetylCoA carboxylase in fat cells has also been demonstrated [16, 17] but the role

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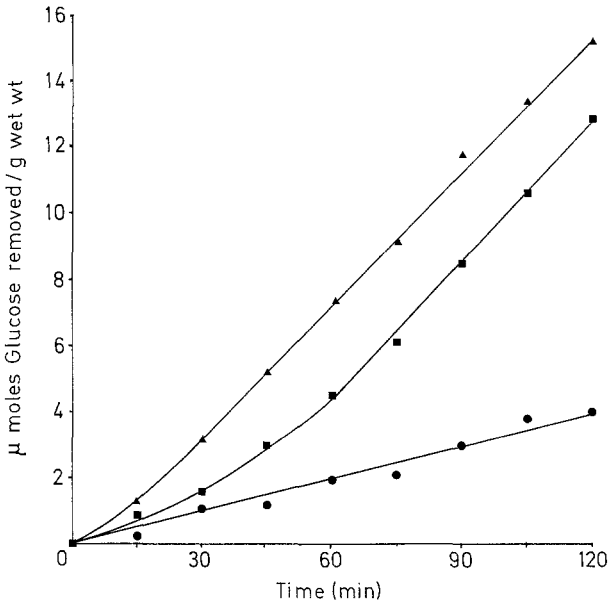

Fig. 1. Time courses of glucose uptake by adipose tissue pieces incubated in Krebs Henseleit bicarbonate medium containing glucose (5 mmol/l) and the following additions: ● none; ▲ insulin (0.5 μg/ml); ■ anti-insulin receptor serum B-2 (1 μl/ml). The pieces were preincubated for 30 min in medium without additions before the start of the incubation period

of changes in phosphorylation in the effects of insulin on the activity of this enzyme has yet to be established [16]. Insulin is also known to cause a marked increase in the phosphorylation of two intracellular proteins of subunit molecular weights of 130,000 [13–16] and 35,000 [12, 15]. During the present studies, the increased phosphorylation of the third protein (subunit molecular weight of 22,000) in fat cells with insulin was recognised.

Materials and Methods

Animals

Epididymal fat pads were obtained from male albino Wistar rats (180–200 g) with free access to water and stock laboratory diet (modified 41B; Oxoid Ltd., London, S.E.1, U.K.).

Serum Containing Anti-Insulin Receptor Antibodies

This was the kind gift of Dr. C. R. Kahn (Diabetes Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014). It had previously been designated serum B-2 [5] and was the same as that used in a number of previous studies with fat cell preparations [4, 6, 7].

Insulin

Stock solutions were prepared by dissolving 0.5 mg/ml bovine insulin (Sigma London Chemical Co. Ltd., Poole, Dorest, U.K.) in 3 mM hydrochloric acid and stored at −20 °C until use.

Measurement of Glucose Uptake and Enzyme Activities in Epididymal Fat Pad Pieces

The pads were cut into small pieces of about 50 mg and incubated at 37 °C in groups of 6 to 8 in 3.5 ml of Krebs-Henseleit bicarbonate buffered medium (pH 7.4 with 1.25 mmol/l $CaCl_2$) containing glucose (5 mmol/l) and appropriate additions of insulin or serum B-2 as indicated and gassed with O_2:CO_2 (95:5).

At the end of the incubation period, samples of medium were treated with perchloric acid (0.1 mmol/l) and assayed for glucose [20]. The pieces of adipose tissue were rapidly frozen with liquid nitrogen immediately on completion of the incubation.

The frozen pieces were then extracted and the initial and total activities of acetylCoA carboxylase (EC 6.4.1.2.) and pyruvate dehydrogenase assayed as described previously [21, 22]. Total activities of the enzymes were taken as the activities in extracts following treatment with citrate or pyruvate dehydrogenase phosphate phosphatase respectively. Enzyme activites have been expressed as munits where one munit is the amount catalysing the conversion of 1 nmol of substrate in 1 min at 30 °C.

Determination of Phosphorylation of Proteins in Isolated Fat Cells

The techniques followed those described previously [15–17]. In brief, isolated fat cells were prepared by collagenase digestion and then pre-incubated with $^{32}P_i$ (0.2 mmol/l and about 1000 dpm/pmol) for 1 h followed by incubation for a further 15 min in the presence of appropriate additions of insulin or serum. The cells were broken by vortexing in cold medium (sucrose (0.25 mol/l) containing Tris (10 mmol/l) ethylene glycol-bis-(amino-ethyl-ether)-tetra-acetate (EGTA) (2 mmol/l) and reduced glutathione (7.5 mmol/l), pH 7.4). Following the removal of fat by brief centrifugation (1000 g/min), extracted proteins were immediately precipitated with trichloracetic acid (10% w/v). These proteins were redissolved in gel buffer which contained sodium dodecylsulphate (SDS) (40 mg/ml), sucrose (200 mg/ml), Tris (62.5 mmol/l), bromophenol blue (0.2 mg/ml) and 2-mercaptoethanol (100 mmol/l), pH 6.8 and heated at 100 °C for 5 min. Separation of the proteins was carried out using SDS-polyacrylamide gel electrophoresis in 1 cm tracks on 10% (w/v) or 12% (w/v) polyacrylamide slab gels (14 cm × 14 cm) using solutions as described by Laemmli [23]. After electrophoresis the proteins were fixed and then stained with Coomassie Blue and the gels laid on cellophane and dried on boards under vacuum. The dried gels were exposed to Kodak Kodirex KT X-ray film and the autoradiographs scanned at 625 nm in a Gilford recording spectrophotometer.

Results

Effects of Anti-Insulin Receptor Serum B-2 on Glucose Uptake by Pieces of Rat Epididymal Adipose Tissue and Isolated Fat Cells

Figure 1 illustrates the effects of insulin and the serum B-2 on the time course of glucose uptake by rat epididymal adipose tissue pieces. The uptake of

Table 1. Effect of insulin and anti-insulin receptor serum B-2 on the uptake of glycose and the initial activities of pyruvate dehydrogenase and acetylCoA carboxylase in rat epididymal adipose tissue pieces

Additions to medium	Glucose uptake (μmol/h/g wet wt)	Enzyme activities (as munit/g wet wt)					
		Pyruvate dehydrogenase			AcetylCoA carboxylase		
		Initial (I)	Total (T)	100 I/T	Initial (I)	Total (T)	100 I/T
None (control)	2.24±0.15 (8)	61.3± 6.5	158±25	41±3.5 (7)	21±2.4	123±16	18±1.2 (8)
Insulin (0.5 μg/ml)	8.55±1.24 (4)[b]	118 ±16[b]	177±32	69±9.3 (4)[b]	43±7.5[b]	137± 7.4	31±4.1 (4)[a]
Anti-insulin receptor serum B-2 (1 μl/ml)	6.34±1.13 (4)[b]	121 ±16[b]	150± 8	80±6.1 (4)[b]	40±3.9[b]	115±21	37±3.7 (4)[b]

Adipose tissue pieces were preincubated for 30 min in medium containing glucose (5 mmol/l) and other additions as indicated and then transferred to fresh medium of the same composition and incubated for a further 60 min. Glucose uptake, pyruvate dehydrogenase and acetylCoA carboxylase activities were determined as described in Methods. All results are shown as mean ± SEM for the number of separate observations given in parenthesis

[a] $P<0.05$; [b] $P<0.01$ versus control value

glucose was increased fourfold by a saturating concentration of insulin (0.5 μg/ml) within 15 min. The addition of serum B-2 (1 μl/ml) leads to a similar maximum stimulation in glucose uptake but this was only achieved after about 1 h of exposure to the serum.

Table 1 shows the effects of the same concentrations of insulin and serum B-2 measured over a 60 min period following a 30 min preincubation period also in the presence of either insulin or serum B-2. Again a fourfold increase in glucose uptake was observed in the presence of insulin. The increase in the presence of the serum B-2 was about three fold, consistent with the time course shown in Figure 1.

Effects of Anti-Insulin Receptor Serum B-2 on the Initial Activities of Pyruvate Dehydrogenase and AcetylCoA Carboxylase

The exposure to insulin resulted in significant increases in the initial activity of both pyruvate dehydrogenase and acetylCoA carboxylase measured in tissue extracts prepared at the end of the incubation period (Table 1). Initial activities were also increased in tissue pieces incubated with serum B-2 (1 μl/ml) by at least the same extent as that observed with insulin (Table 1). The actual percentage increases in initial activities of pyruvate dehydrogenase and acetylCoA carboxylase (based on paired control values) with insulin were 92 ± 30 (4) and 78 ± 23 (4) respectively whereas those in the presence of serum B-2 (1 μl/ml) were 111 ± 31 (3) and 117 ± 33 (4) respectively. A second similar series of experiments were conducted using 0.5 μl/ml of the serum. The percentage increases in initial activities of pyruvate dehydrogenase and acetylCoA carboxylase with insulin (0.5 μg/ml) were 87 ± 19 (8) and 125 ± 51 (7) respectively whereas those in the presence of serum B-2 (0.5 μl/ml) were 53 ± 10 (7) and 79 ± 19 (7). Results are given as mean ± SEM for the number of paired observations given in parenthesis.

No significant changes in the total activities of either enzyme were observed with insulin or serum B-2 (Table 1).

Effects of Anti-Insulin Receptor Serum B-2 on the Phosphorylation of Proteins in Isolated Fat Cells

Figures 2a and 3 (trace a) show the densitometric scans of autoradiographs of [^{32}P] labelled phosphoproteins from fat cells incubated under standard conditions in the presence of $^{32}P_i$ for 75 min. The phosphoproteins were separated by SDS-polyacrylamide gel electrophoresis. The numbers of the bands given in the figures correspond to those given in previous studies using phosphate gels with 7.5% polyacrylamide and the period of incubation was sufficient to achieve their maximum steady-state labelling [15, 16]. In the present studies, the labelled phosphoprotein band originally designated band 1 (which is largely acetylCoA carboxylase) was not well separated from the interface between the spacer 4% gel and the separating 10 or 12% gel. On the other hand, these systems revealed additional bands representing phosphoprotein with subunit molecular weights of about 22,000 and 18,000 which were not separated from the solvent front in our earlier studies. These we have designated band 9 and band 10. In particular the 12% gel clearly resolved band 7 and band 9 and enabled the changes in these bands to be seen more clearly (Figs. 2 and 3).

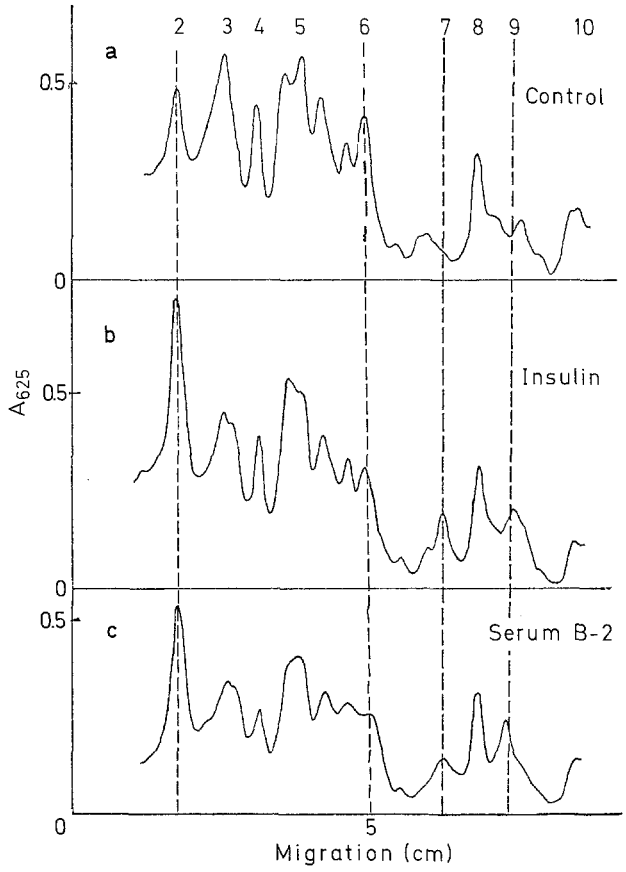

Fig. 2. Densitometric traces of autoradiographs of [^{32}P] labelled phosphoproteins from fat cells separated by SDS-polyacryamide (10% w/v) slab-gel electrophoresis. Fat cells were incubated for 1 h in medium containing [^{32}P] phosphate, albumin (10 mg/ml) and glucose (1 mM) and then for a further 15 min in the same medium with the following additions: (a) none; (b) insulin (0.5 μg/ml); (c) anti-insulin receptor serum B-2 (1 μl/ml). The dye front (bromophenol blue) was allowed to migrate 10 cm. Results are typical of experiments carried out on three different preparations of cells. Also indicated are the protein band numbers used in the text

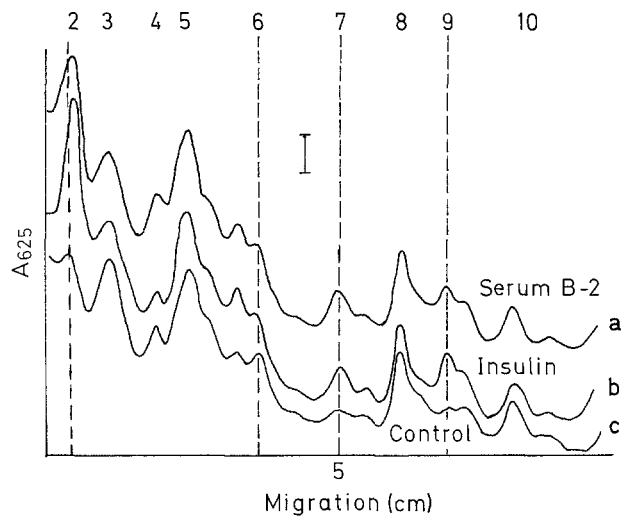

Fig. 3. Experimental details are the same as for Figure 1. Except that a 12% (w/v) polyacrylamide gel was used. This separation was carried out on samples from the same experiment as shown in Figure 1. The baselines of the different traces have been separated for the sake of clarity. The bar represents $^{A}625$ nm = 0.2. Additions to the cells were: (a) none (b) insulin (0.5 μg/ml) (c) anti-insulin receptor serum B-2 (1 μl/ml)

It can be seen from Figures 2b and 3 (trace b) that when insulin was added to the incubation medium for the last 15 min the extent of phosphorylation of bands 2 and 7 was greatly increased. These bands have subunit molecular weights of approximately 130,000 and 35,000 respectively. It is also evident that the phosphorylation of a further smaller protein was increased. This is the band designated 9 and has a subunit molecular weight of about 22,000. On the other hand, exposure to insulin also resulted in a small descrease in the phosphorylation of band 6 (which contains the phosphorylated α-subunit of pyruvate dehydrogenase [12, 15].

Figures 2c and 3 (trace c) show the scans of the labelled phosphoprotein in cells exposed to 1 μl/ml serum B-2 for 15 min. As with insulin, the phosphorylation of bands 2, 7 and 9 were all clearly increased whereas that of band 6 was decreased. The traces shown in Figures 2 and 3 are taken from one of three similar experiments with different batches of cells. In all cases the serum (1 μl/ml) resulted in changes in bands 2, 6, 7 and 9 which were similar to those observed with insulin.

Discussion

Previous studies on the effects of the serum B-2 on glucose uptake, glucose metabolism, glycogen synthase activity and phosphorylase activity in fat cells showed that maximum effects were observed at about 1 μl/ml [4, 6]. At this concentration the changes observed with the serum B-2 were as large as those observed with saturating concentrations of insulin, were not effected by the presence of anti-insulin serum but were largely lost on the addition of anti-IgG serum [4, 6]. No additional effects of insulin were observed in the presence of 1 μl/ml of the serum B-2 [6].

The present studies demonstrate that the serum B-2 at the concentration of 1 μl/ml also causes increases in the initial activities of pyruvate dehydrogenase (a mitochondrial enzyme) and acetylCoA carboxylase (a cytoplasmic enzyme) in adipose tissue pieces (Table 1). These increases were at least as

large as those observed with saturating concentrations of insulin. There was a considerable lag period before the maximum effect of serum B-2 was observed on glucose uptake by adipose tissue pieces. We presume this lag reflects the slow penetration by the antibodies into the tissue pieces compared to insulin. No such lag period appeared to exist in the effects of the serum B-2 on isolated fat cells. Changes in the phosphorylation of proteins similar to those observed with insulin in isolated fat cells were evident after 15 min. These changes included a decrease in the phosphorylation of band 6 (which contains the α-subunit of pyruvate dehydrogenase) and increases in the phosphorylation of bands 2, 7 and 9. The effect of insulin on the phosphorylation of band 6 was smaller than that found previously [12, 15]. With the batches of collagenase available to us at the time of these studies, pyruvate dehydrogenase in the fat cells appeared to be largely dephosphorylated and thus the initial activity was close to the total activity even in the absence of insulin. We have encountered this problem before [24].

Taken together with the studies of Lawrence et al. [4], our results suggest that the serum B-2 may be capable of initiating all the short term intracellular effects of insulin in adipose tissue. All the effects studied appear to be largely independent of the stimulation of glucose transport [4, 11, unpublished observations by R. W. B. and W. A. H.]. The findings lend further support to the view that the short-term intracellular effects of insulin are bought about by mechanisms which do involve transfer of the insulin molecule, or part thereof, into cells. It would appear that the interaction of insulin or anti-insulin receptor antibodies with the insulin receptors in the cell membrane bring about changes, perhaps including aggregation [25], in the insulin receptor which initiate the generation of an unknown signal (or signals) within the cells which in turn leads to the observed intracellular events.

Evidence for the internalization of ^{125}I-insulin into liver cells has been recently reported but since much of the labelled insulin was found associated with the lysosomes this may be a degradative pathway [26]. Insulin binding has been observed with preparations of nuclei and other intracellular organelles from rat liver [2]. However, serum B-2 does not inhibit the binding of insulin to the nuclei and thus it would seem unlikely that the antibodies could elicit any biological activity at these sites [2].

The present studies also serve to underline that the effects of insulin on cells probably involve increases in phosphorylation of certain proteins as well as the dephosphorylation of others, such as pyruvate dehydrogenase and glycogen synthase. The physiological importance of the phosphorylation of the three proteins showing increased phosphorylation after 15 min exposure of fat cells to insulin (or the serum B-2) has not been fully characterized. The protein of subunit molecular weight 130,000 may be ATP-citrate lyase [27, 28] and that of 35,000 may be a ribosomal protein [11, 29]. The smallest of the three phospho-proteins (band 9; subunit molecular weight 22,000), which was first recognised during the course of these studies, may be the inhibitor protein, known as I_1 of the general phosphoprotein phosphatase [18, 19]. This protein becomes a potent inhibitor of the phosphatase when phosphorylated on a specific threonine residue by cyclic AMP dependent protein kinase [18, 19, 29]. Evidence that band 9 may represent the phosphorylation of this protein may be summarised as follows: (i), the apparent subunit molecular weight on SDS-polyacrylamide gel electrophoresis; (ii), the phosphorylation of the protein in fat cells is also brought about by adrenaline (iii), the protein is the only labelled phosphoprotein which is not precipitated with 2% trichloroacetic acid or by heating at 100 °C for 10 min, but is precipitated by 10–15% trichloroacetic acid [unpublished observations of G. J. B.].

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