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Catecholamines and tumour promoting phorbolesters inhibit insulin receptor kinase and induce insulin resistance in isolated human adipocytes

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Summary. The effect of the catecholamine isoprenaline (10^{-5} mol/l) and of the tumour promoting phorbolester tetradecanoyl-β-phorbol acetate (10^{-9} mol/l) on insulin stimulated 3-O-methyl-glucose transport was studied in freshly isolated human adipocytes. Both substances reduced the maximal responsiveness of the glucose transport system to insulin by approximately 50%. To test if this is caused by inhibition of the insulin receptor kinase the receptor from phorbolester and isoprenaline treated cells was solubilized, partially purified and its kinase activity studied in vitro. Insulin stimulated ^{32}P-incorporation into the β-subunit of the insulin receptor of phorbolester or isoprenaline treated cells was reduced to 20–60% of the values found with receptor from control cells at insulin concentrations between 10^{-10} mol/l and 10^{-7} mol/l. This inhibition of kinase activity of receptor from phorbolester and isoprenaline treated cells was observed at nonsaturating adenosine triphosphate levels (5 µmol/l), and it could be overcome with higher concentrations of γ-^{32}P-adenosine triphosphate in the phosphorylation assay. A Lineweaver Burk analysis of the insulin stimulated receptor phosphorylation revealed that the Michaelis constant for adenosine triphosphate of the receptor kinase from phorbolester and isoprenaline treated cells was increased to >100 µmol/l compared with <50 µmol/l for receptor from control cells. We conclude from the data that catecholamine and phorbolester treatment of human adipocytes modulates the kinase activity of the insulin receptor by increasing its Michaelis constant for adenosine-triphosphate, and propose that this modulation of receptor kinase is a mechanism that can contribute to the pathogenesis of insulin resistance in human fat cells.

Key words: Insulin receptor kinase, insulin resistance, glucose transport, catecholamines, phorbolester.

Catecholamines induce insulin resistance in man [1, 2], and it appears that this insulin resistance arises from a direct antagonism of catecholamines and insulin at the insulin sensitive tissues [3]. The cellular reactions induced by catecholamines causing insulin resistance are at present not understood in detail. It is known that catecholamine treatment of isolated rat adipocytes [4–6] and human fat cells [5] induces insulin resistance of the glucose transport system. This insulin resistance seems to be caused by an inhibitory effect of catecholamines on insulin signal transmission predominantly at a postbinding site [4]. However, the exact molecular mechanism of the inhibition of insulin signal transmission is not known.

A similar pattern of cellular insulin resistance found after catecholamine treatment can be induced by treatment of rat adipocytes with the tumour promoting phorbolester tetradecanoyl-β-phorbol acetate (TPA) [7]. Using rat fat cells we were recently able to show that both substances modulate the insulin receptor tyrosine kinase [8, 9], and have proposed that they induce insulin resistance through inhibition of the receptor kinase in the rat fat cell. Cellular insulin action is initiated by binding of the hormone to the α-subunit of the receptor. This stimulates the phosphorylation of the β-subunit of the insulin receptor in intact cells [10–13] or solubilized receptor preparations [14–21]. It is clear now that this insulin stimulated phosphorylation is due to the activation of a tyrosine kinase in the β-subunit of the insulin receptor [19]. This kinase undergoes insulin stimulated autophosphorylation at tyrosine residues; it is believed that insulin stimulated autophosphorylation of the receptor kinase is the first postbinding step in insulin signal transmission across the plasma membrane.

The aim of the present study was to investigate if catecholamines and phorbolesters modulate the insulin receptor kinase in the human fat cell as well. We found that both substances rapidly induce insulin resistance of the glucose transport system in isolated human fat cells. The insulin receptor kinase isolated from these cells in

vitro shows a reduced activity which is apparently due to a modulation of the adenosine triphosphate (ATP)-binding site of the insulin receptor kinase. We speculate that this modulation of the ATP-binding site occurs through stimulation of serine phosphorylation of the insulin receptor. Further, it seems possible that this mechanism of receptor kinase inhibition plays an important role in the pathogenesis of insulin resistance in human fat cells.

Materials and methods

Materials

Porcine [^{125}J-TyrA14] insulin and [γ-^{32}P]-ATP (2900 Ci/mmol), were from New England Nuclear (Dreieich, FRG); aprotinin, phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, benzamidin, bacitracin and leucin were from Sigma (Munich, FRG). Wheat germ agglutine coupled to agarose was from Miles (Munich, FRG); Triton X-100, all reagents for Dodecyl sodium sulphate SDS polyacrylamide gel electrophoresis and the protein assay were from Bio-Rad (Munich, FRG); and all reagents were of the best grade commercially available.

Methods

150 g of fresh subcutaneous fat were obtained from four patients undergoing abdominal plastic surgery. Full informed consent was obtained in each case. Isolated fat cells were prepared by the collagenase method [22] as described earlier for rat fat cells [23]. The cells of each patient (120–160 ml of approximately $4–5 \times 10^6$ cells/ml) were divided into three aliquots which were incubated with isoprenaline (10^{-5} mol/l), TPA (10^{-9} mol/l) or buffer containing glucose (15 mmol/l) alone for 20 min. Subsequently the insulin effect on glucose transport was determined. In only three of the four patients were sufficient amounts of fat cells obtained to prepare partially purified insulin receptor from each group.

3-O-Methylglucose transport in adipocytes

3-O-Methylglucose transport was measured as previously described [23]. Aliquots (100 µl) of the concentrated cell suspension (5×10^6 cells/ml) were drawn together with 200 µl 3-O-methylglucose (final concentration 0.5 mmol/l) and a tracer of 0.1 µCi 3-O-methyl-D-[^{14}C] glucose (Radiochemical Center, Amersham, UK) into a mixing pipette (Gilson Medical Electronics, Villiers le Bel, France). After 4 s, the uptake was stopped by diluting the cells in 5 ml NaCl (0.9 g/dl) which contained phloretin (1 mmol/l). Cells and medium were separated by centrifugation through silicone oil (1000 *g* for 60 s). The cell layer was removed by a pipette, added to scintillation fluid and the radioactivity measured. The amount of 3-O-methylglucose in extracellular fluid or taken up by diffusion was determined in samples which contained 1 mmol/l phloretin throughout. All other uptake data were corrected by this value. 3-O-methylglucose accumulation measured during 4 s is expressed as a percentage of the 3-O-methylglucose accumulated at equilibrium in fat cells after 30 min incubation at saturating insulin concentrations (67 nmol/l).

Partial purification and phosphorylation of insulin receptor

The receptor was prepared as described earlier for isolated rat adipocytes [12], with some of our recently reported modifications [24]. Isolated cells were lysed by repeated freezing and thawing in the presence of protease inhibitors, PMSF (5 mmol/l), aprotinin (1200 trypsin inhibiting units [TIU]/l), leupeptin (2 µmol/l), pepstatin (2 µmol/l), benzamidin (10 mmol/l), bacitracin (7500 U/l), leucin (10 mmol/l) and EDTA (5 mmol/l) in a buffer containing NaH_2PO_4 (10 mmol/l) and then homogenized by an ultra-turrax for 10–15 s with maximal speed at 22 °C. The following steps were carried out at 4 °C. The homogenates were centrifuged at 200,000 *g* for 50 min; the pellet was then solubilized with Triton X-100 at a final concentration of 1% (by vol.) in a buffer containing Hepes (25 mmol/l) and the protease inhibitors aprotinin (600 TIU/l), leupeptin (2 µmol/l), pepstatin (2 µmol/l) and PMSF (2 mmol/l). Insoluble material was removed by centrifugation at 200,000 *g* for 50 min and the clear supernatant with addition of the same volume 25 mmol/l HEPES and 150 mmol/l NaCl was applied to columns (1 × 2.5 cm) of wheat germ agglutine coupled to agarose. After washing with buffer containing 25 mmol/l Hepes and 0.1% (by vol.) Triton X-100 the specifically bound material was eluted with 10 ml of the same buffer supplemented with 0.3 mmol/l N-acetylglucosamine (flow rate: 50–60 ml/h). 0.5 ml fractions of the eluate were collected and protein content determined by a protein assay from Biorad and the insulin binding as described below. The fractions containing most protein were used for further studies. For the standard phosphorylation assay approximately 5 µg of wheat germ purified proteins were preincubated at 22 °C for 30 min with insulin (10^{-10} mol/l–10^{-6} mol/l) or without insulin. This was followed by an incubation with [γ-^{32}P]-ATP (5 µmol/l, 0.01 mCi), if not otherwise stated, in 25 mmol/l Hepes buffer, containing 0.1% (by vol.) Triton X-100, 10 mmol/l $MnCl_2$, 12 mmol/l NaCl; 0.5 mmol/l KCl; 0.25 mmol/l $CaCl_2$, 0.1 mmol/l KH_2PO_4, 0.1mmol/l $MgSO_4$, 0.25g% bovine serum albumin (BSA), pH 7.4 at 22 °C, and 1 mmol/l vanadate at 22 °C for the times indicated in the figure legends. The incubation was stopped by addition of Laemmli buffer [25] containing 100 mmol/l dithiotreitol and boiling for 30 min. Subsequently phosphoproteins were separated by polyacrylamide gel electrophoresis and identified by autoradiography. The phosphoproteins identified by the autoradiography were cut from the gel and counted in a scintillation counter.

Binding to solubilized receptor

Solubilized and wheat germ purified receptor was prepared as described above. Aliquots of wheat germ eluate containing 5 µg protein were incubated with mono-A14[^{125}I]-insulin (3×10^{-11} mol/l) and various concentrations of unlabelled insulin for 45 min at 22 °C in a medium of 5 mmol/l $MgSO_4$ and 0.5g% BSA/Tris 25 mmol/l at pH 7.4. Separation of the free and receptor-bound insulin was then performed using dextrane-coated charchoal.

Results

Glucose transport

Freshly isolated human adipocytes were incubated for 20 min with isoprenaline (10^{-5} mol/l) or TPA (10^{-9} mol/l). Subsequently the insulin effect on 3-O-methyl-glucose transport was measured in these cells and control cells. Figure 1 shows the effect of isoprenaline (10^{-5} mol/l) or TPA (10^{-9} mol/l) on the insulin stimulation of 3-O-methyl glucose transport. As described earlier for the rat fat cell [4–7], a reduced maximal responsiveness of the glucose transport to insulin was found with both substances.

Insulin binding

We isolated insulin receptor from control, isoprenaline and TPA treated cells as described in the methods sec-

tion and determined insulin binding and kinase activity of the solubilized receptor. Figure 2 shows the binding of ^{125}I-insulin to solubilized and partially purified insulin receptor. In contrast to our earlier findings in the rat [8, 9], no difference of insulin binding to the solubilized insulin receptor from catecholamine or TPA treated cells and control cells was found. This difference to the earlier findings in the rat is not explained by altered experimental conditions and suggests, therefore, that the catecholamine effect and the phorbol ester effect on insulin binding might be species specific. Aliquots of 5 µg glycoproteins from control cells, TPA and isoprenaline-treated cells each have a binding capacity of approximately 4 fmol insulin. The Scatchard plot suggests that there is also no difference in the affinity of the binding sites.

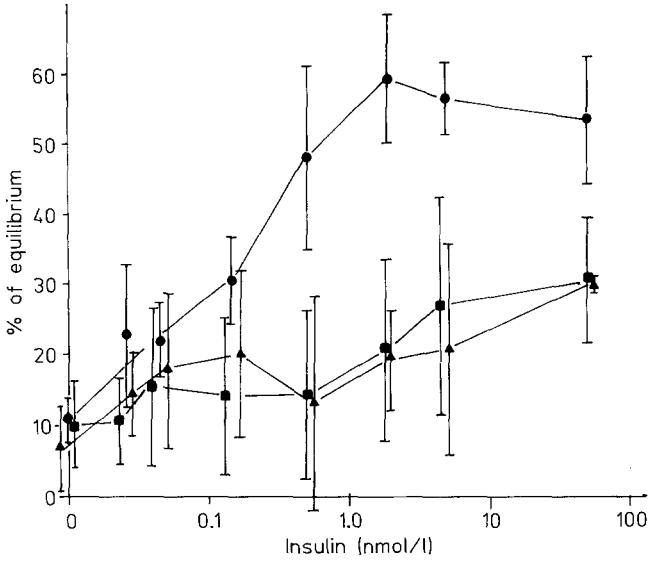

Fig. 1. Effect of isoprenaline treatment and tetradecanoyl-β-phorbol acetate (TPA) treatment on insulin stimulation of D-glucose transport. Human adipocytes were preincubated for 20 min at 37 °C in the absence (control) (●) or the presence of isoprenaline (10^{-5} mol/l) (▲) or TPA (10^{-9} mol/l) (■). Subsequently, insulin was added for 20 min in the concentrations given on the abscissa. D-glucose uptake was measured with 3-O-methyl-D-^{14}C-glucose as described in the Methods section and expressed as a percentage of equilibrium values obtained at saturating insulin concentrations after 20 min. The values represent the means + SD of 4 independently performed experiments

Receptor phosphorylation

Insulin stimulated receptor kinase activity was studied with γ-^{32}P ATP and insulin as described in the methods section. The autoradiogram in Figure 3 shows phosphorylated proteins from control cells. When the glycoprotein fraction was preincubated with or without insulin (10^{-7} mol/l) and then phosphorylated, a major phosphorylation of a 95 kDa band was found (Fig. 3, lane A). Insulin (10^{-7} mol/l) stimulated the ^{32}P-incorporation into the 95 kDa band approximately 3-fold (Fig. 3, lane B). If the phosphorylated proteins were immunoprecipitated with serum containing insulin receptor antibody as described in the legend of Figure 3, the 95 kDa protein was quantitatively precipitated (Fig. 3, lane C and E). This identifies the phosphorylated 95 kDa protein as the β-subunit of the insulin receptor of human fat cells [24]. Equal amounts of protein from each preparation representing equal amounts of insulin receptors as determined by the binding values were used. To compare receptor phosphorylation in wheat germ purified proteins from control cells, isoprenaline or TPA treated cells, samples of 5 µg protein were preincubated without or with insulin (10^{-10} mol/l–10^{-7} mol/l) for 30 min followed by a 15 min phosphorylation with γ-[^{32}P]-ATP (5 µmol/l). Figure 4 shows autoradiograms demonstrating the effect of increasing insulin concentrations (10^{-9} mol/l–10^{-7} mol/l) on the receptor phosphorylation. The autoradiogram of phosphoproteins from control cells is shown in the middle, the one from isoprenaline treated cells on the right side of Figure 4. The autoradiogram of receptor from TPA treated cells is not shown, as it is essentially identical to the autoradiogram

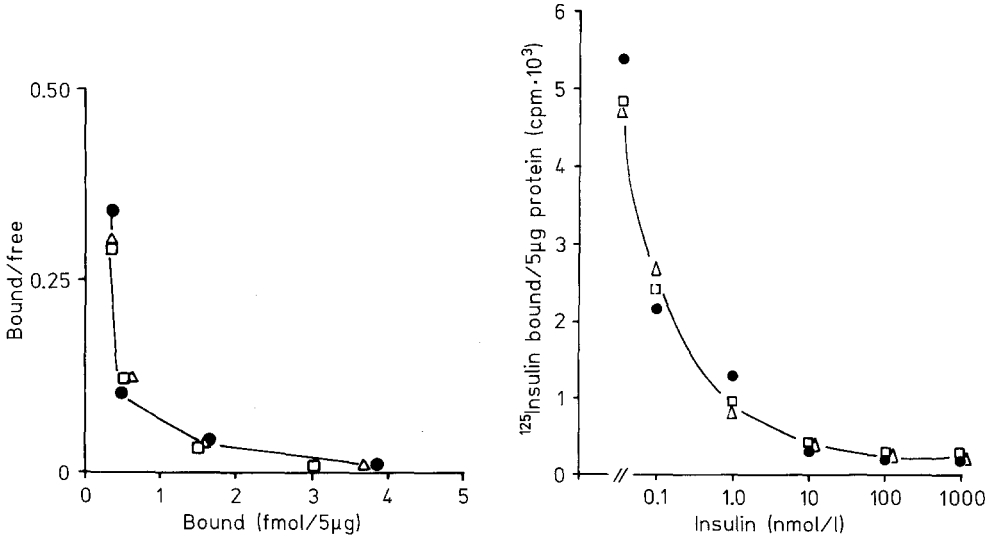

Fig. 2. Insulin binding to partially purified receptor from isoprenaline and TPA treated cells. Right side: Solubilized and partially purified insulin receptor (5 µg protein) from isoprenaline (10^{-5} mol/l) treated cells (△), TPA treated cells (□) or control cells (●) were incubated with 3×10^{-11} mol/l A14-[^{125}I]-monoinsulin together with increasing amounts of unlabelled insulin as described in the Methods section. Displacement curves are shown. Left side: Scatchard plot of the insulin binding to receptor from control (●), TPA (□) and isoprenaline treated cells (△)

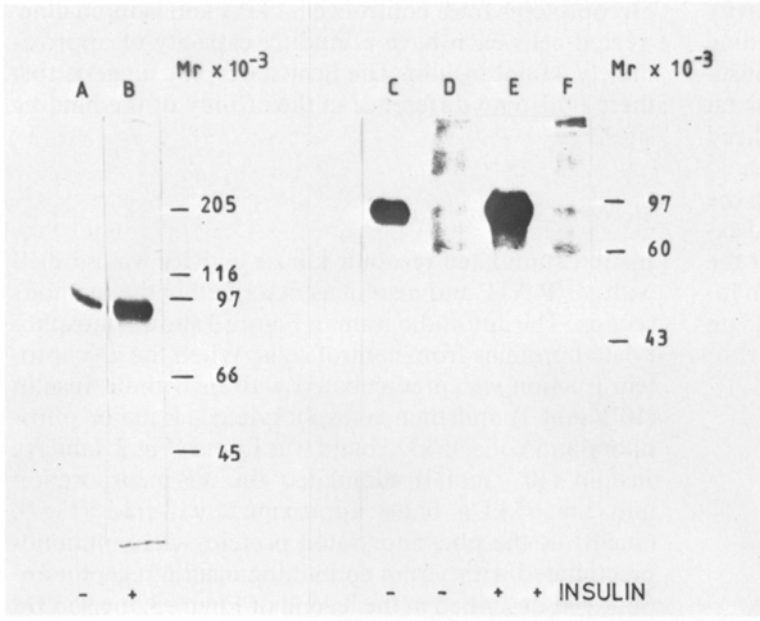

Fig. 3. Autoradiogram of the phosphorylated insulin receptor from human fat cells. Wheat germ affinity chromatography-purified receptor from human fat cells was incubated for 30 min at room temperature with insulin (10^{-7} mol/l). Phosphorylation was studied at 22 °C with [^{32}P] ATP (5 µmol/l, 0.01 mCi) for 15 min. 5 µg protein was used in each assay. The reaction was stopped by boiling in Laemmli buffer. Phosphoproteins were analysed by Na DodSPO$_4$ 7.5% polyacrylamide gel electrophoresis. Lane A shows phosphorylation in the absence of insulin, lane B in the presence of insulin. In the experiments shown in lanes C to F, phosphorylation was not stopped by boiling but by addition of a stopping solution (200 mmol/l NaF, 20 mmol/l sodium pyrophosphate, 10 mmol/l EDTA, 10 mmol/l ATP, 0.4 mmol/l vanadate and 2400 TIU/l aprotinin). The phosphoproteins were then immunoprecipitated with insulin receptor antibody containing serum. Lane C shows the immunoprecipitate without and lane E with stimulation of the autophosphorylation by insulin. Lane D and F show the supernatant of the immunoprecipitates. Immunoprecipitates were analyzed by NaDodSO$_4$ 10% polyacrylamide gel electrophoresis

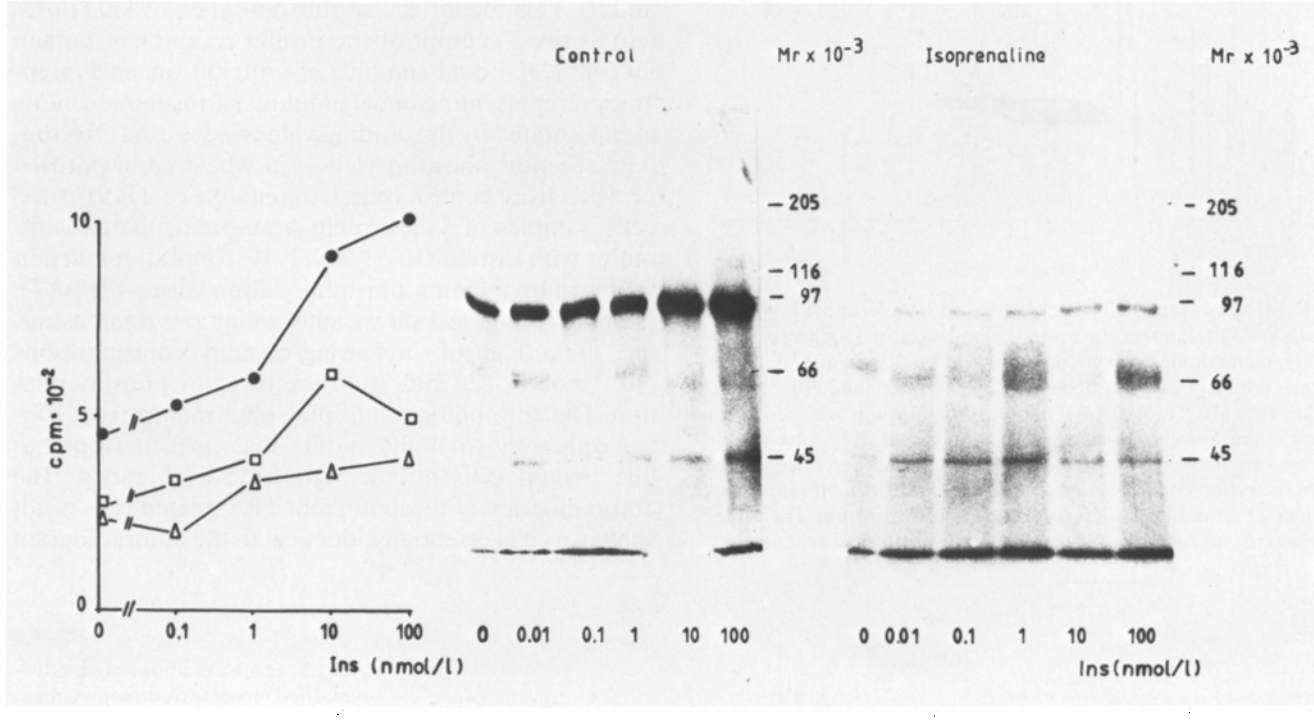

Fig. 4. The effect of the insulin concentration on the equilibrium phosphorylation of the 95 kDa subunit of the insulin receptor from TPA, isoprenaline and control cells. Following a 30-min incubation with the indicated concentrations of insulin the solubilized insulin receptor (5 µg of protein) was incubated during a 15 min time interval with 5 µM γ-^{32}P-ATP. The autoradiogram in the middle shows receptor from control cells; the receptor from isoprenaline treated cells is shown on the right. The autoradiogram of receptor from TPA treated cells is not shown, as it is essentially identical to the autoradiogram of receptor from isoprenaline treated cells. The 95 kDa bands identified on the autoradiographies were cut from the gel and the radioactivity was counted. The graph shows the mean values of three experiments for receptor from control (●), TPA (△) and isoprenaline (□) treated cells. Under maximal insulin stimulation (10^{7} mol/l) the inhibition caused by isoprenaline, respectively, TPA was as follows: Exp. 1, 48% ISO, 36% TPA. Exp. 2, 55% ISO, 51% TPA. Exp. 3, 77% ISO, 63% TPA

of receptor from isoprenaline treated cells. The 95 kDa band was cut from the gel and the ^{32}P incorporation was counted in a scintillation counter. This leads to the dose response curves of the insulin effect on receptor phosphorylation shown in the graph at the left side of Figure 4. The maximally effective insulin concentration (10^{-7} mol/l) stimulated the phosphorylation of receptor from control cells in three experiments 2.3-fold, while the stimulation factor with receptor from TPA or isoprenaline treated cells was only 1.4-fold. The graph

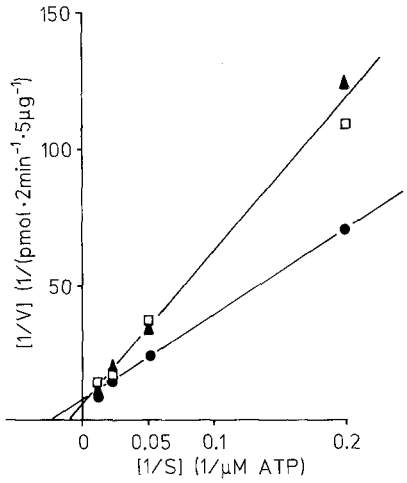

Fig. 5. The effect of the ATP concentration on initial rates of autophosphorylation of the 95 kDa subunit of the insulin receptor from isoprenaline (▲) treated, TPA (□) treated and control cells (●). Solubilized insulin receptor (5 µg) was incubated with 10^{-7} mol/l insulin for 30 min followed by the addition of γ-^{32}P-ATP in the concentrations indicated on the abscissa. The amount of tracer was kept constant at all ATP concentrations. The reaction was stopped after 2 min, the 95 kDa band was identified on the autoradiogram and the band was cut from the gel and the radioactivity was counted. The ^{32}P incorporation per 2 min was calculated and the data expressed in a Lineweaver Burk plot. Mean values of three receptor preparations are shown

on the left side of Figure 4 and the values given in the legend of Figure 4 show, furthermore, that the insulin stimulated ^{32}P incorporation into the 95 kDa subunit from TPA and isoprenaline treated cells is clearly reduced compared to the values found with the insulin receptor from control cells at all insulin concentrations tested, suggesting a decreased kinase activity of the insulin receptor from isoprenaline and TPA treated cells. To test if the decreased autophosphorylation of the receptor from TPA and isoprenaline treated cells might reflect a decreased affinity of the receptor kinase for ATP as found in our earlier studies with isoprenaline and phorbolester treated rat adipocytes [8, 9], we studied the effect of the ATP concentration on the autophosphorylation of receptor from each group. In order to determine the initial rates of receptor phosphorylation, the ^{32}P incorporation within 2 min after addition of ^{32}P-ATP was measured. Figure 5 shows a Lineweaver Burk plot of the insulin stimulated phosphorylation rates of the 95 kDa receptor subunit. The V_{max} of both receptor preparations appears similar while the K_m for ATP of the receptor kinase from isoprenaline and TPA treated cells is >100 µmol/l compared to <50 µmol/l for the receptor of control cells.

Discussion

Catecholamines and phorbol esters both inhibit the insulin stimulation of glucose transport in human adipocytes. Furthermore, both substances inhibit the kinase activity of the insulin receptor; it appears that this is due to a modulation of the ATP binding site of the insulin receptor kinase. The data suggest that inhibition of the insulin receptor kinase might be one important mechanism in the pathogenesis of cellular insulin resistance in human target cells of insulin action.

The correlation of insulin receptor kinase activity and cellular insulin effects like the regulation of glucose transport, glycogen synthesis, TAT-induction and cell growth was studied in a number of insulin resistant cell models derived from animals. In several of these cell models a loss of the insulin effect was paralleled by a reduced receptor kinase activity, suggesting that defects of the receptor kinase are a possible cause of cellular insulin resistance [26–29, 8, 9, 32]. In contrast, information about the coincidence of insulin resistance and kinase defects in man are rare. Two patients with the type A syndrome of extreme insulin resistance were found which had apparently a specific defect of the receptor kinase of their monocytes [34], or respectively erythrocytes and fibroblasts [33]. In contrast, the majority of the patients with this syndrome had no defect of their tyrosine kinase activity [35], suggesting that a genetic defect of the receptor kinase is probably very rarely a cause of cellular insulin resistance in man. The catecholamine and phorbol ester induced inhibition of the receptor kinase in human fat cells is an example of a functional inhibition of the receptor kinase leading to insulin resistance. It seems possible that functional defects of the receptor kinase might be more common than genetic defects and it might be speculated that this type of receptor kinase inhibition could also be involved in the pathogenesis of insulin resistance in Type 2 (non-insulin-dependent) diabetes.

Concerning the mechanism of receptor kinase inhibition by catecholamines and phorbolesters two major questions arise. First, is it likely that the increase of the K_m for ATP observed here in vitro leads to a decreased tyrosine kinase activity in the intact cell. As already discussed elsewhere [8, 9] the ATP concentration calculated from the water space of intact adipocytes is in the millimolar range [23, 41]. Considering this ATP concentration in the intact cell and the K_m values found here for the receptor kinase in vitro, it seems unlikely that the isoprenaline- or TPA-induced increase of the K_m for ATP leads to a decreased receptor kinase activity in the intact cell. However, the K_m values were determined in vitro in the presence of 10 mmol/l Mn^{2+}, which specifically decreases the K_m for ATP [42]; these conditions were necessary in our experiments, as the concentration of the receptor isolated from the human fat cells was extremely low. To be able to detect sufficient ^{32}P incorporation at physiological insulin concentrations it was crucial to use conditions where effects of isotope dilution are minimized. Higher K_m values are found at low Mn^{2+} concentrations, which are more likely to resemble the situation in the intact cell [42]. Presuming an analogous modulation of the K_m of the receptor kinase

by isoprenaline and TPA takes also place at the higher K_m-values in the intact cell, it seems possible that this mechanism contributes to the pathogenesis of the insulin resistance observed here. It is clear, however, that an additional defect on the postkinase level of insulin transmission might as well contribute to the insulin resistance of the glucose transport system.

The second question concerns the mechanism of the K_m modulation by catecholamines and phorbolesters. There is some evidence that phorbol esters and catecholamines induce a phosphorylation of the insulin receptor on serine residues [31, 32, 36-38, 43, 44]. While the phosphorylation of the isolated insulin receptor occurs exclusively at tyrosine residues [13-20], a serine phosphorylation of the receptor is found as well if the intact cell is studied [11, 30]. This has suggested that the receptor in the intact cell is also substrate for at least one so far unidentified serine kinase. While tyrosine phosphorylation appears to activate the insulin receptor kinase for other substrates [21], the effect of the serine phosphorylation of the insulin receptor is still open to speculation. Phorbol esters and catecholamines are both stimulators of serine specific kinases in the cell, namely the protein kinase C [39, 40] and the cAMP dependent kinase. There is some evidence that these kinases indeed can phosphorylate the insulin receptor [31, 32, 43, 44]. Therefore, it can be assumed that, in human fat cells as well, stimulation of these kinases leads to serine phosphorylation of the insulin receptor. We believe that this is the mechanism that alters the characteristics of the insulin receptor tyrosine kinase described above. It is tempting to speculate that serine phosphorylation of the insulin receptor kinase might be an important regulatory mechanism which determines the efficiency of the signal transmitting function of the insulin receptor in human target tissues. It will be interesting to study if, in cells of Type 2 diabetic patients, an analogous modulation of the insulin receptor kinase can be found.

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