Insulin receptor kinase in human skeletal muscle

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Receptor-associated protein kinase activity has been shown in all primary target tissues of insulin action in the rat and a function of insulin receptor phosphorylation in signal transmission was proposed. Insulin receptor phosphorylation so far has not been demonstrated in human target tissues of insulin. We describe here insulin receptor kinase activity in human skeletal muscle. Insulin (10^{-8} mol/l) stimulates the phosphorylation of a 95-kDa protein from skeletal muscle 2-fold. The phosphoprotein is quantitatively immunoprecipitated with insulin receptor antibody identifying it as the β-subunit of the insulin receptor. The insulin stimulation of phosphorylation is detectable also at physiological insulin concentrations (10^{-9} mol/l) showing that receptor phosphorylation could be involved in insulin action in human skeletal muscle as well.

Human skeletal muscle *Insulin receptor* *Receptor kinase* *Receptor phosphorylation*

1. INTRODUCTION

Insulin-stimulated phosphorylation of the 95-kDa β-subunit of the insulin receptor was first demonstrated in 1982 [1]. This phenomenon has since been intensively studied [2–10]. The characteristics of insulin stimulation of the insulin receptor kinase and of the phosphorylation of the insulin receptor in the main target tissues of the metabolic effects of insulin in rats, namely liver [2,4,7–9], adipose tissue [3] and the muscle [10] have suggested that the increase of the receptor kinase activity might be the first post-binding step in cellular insulin action which transmits the insulin signal across the plasma membrane. Insulin receptor kinase activity has thus far not been demonstrated in the main target tissues of insulin action in man. Here we studied insulin receptor phosphorylation in human skeletal muscle for two reasons: (i) Receptor kinase activity and its stimulation by physiological insulin concentrations has to be demonstrated in human target tissues to be able to discuss receptor phosphorylation as part of the mechanism of insulin signal transmission in humans. (ii) Insulin resistance in type-II diabetes appears to be caused predominantly by a post-binding defect of insulin action in muscle [11]. Impaired insulin receptor kinase activity is a possible cause of insulin resistance [12–14]. Therefore, it is of great interest for the further characterization of the post-binding defect in type-II diabetes if a measurement of receptor kinase activity is feasible in small amounts of skeletal muscle which could be obtained from patients.

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2. MATERIALS AND METHODS

2.1. *Materials*

Porcine insulin was purchased from Novo Industrie (Denmark), [^{32}P]ATP (2900 Ci/mmol) and Triton X-100 were from NEN (FRG), aprotinin, phenylmethylsulfonyl fluoride (PMSF), leupeptin, and pepstatin were from Sigma (FRG). Wheat germ agglutinin coupled to agarose was from Miles, all reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad, all other reagents were of the best grade commercially available.

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2.2. *Methods*

Human skeletal muscle was obtained from 5 patients undergoing plastic reconstruction of the breast by myocutaneous flap. 2–5 g fresh muscle were used for each preparation. The receptor was prepared as described for rat skeletal muscle [10]. Briefly, skeletal muscle tissue was homogenized by an ultraturrax in the presence of the protease inhibitors PMSF (5 mmol/l), aprotinin (1200 trypsin inhibiting units/l), leupeptin (2 μmol/l), pepstatin (2 μmol/l), benzamidine (10 mmol/l), bacitracin (7500 U/l), leucine (10 mmol/l) in a buffer containing NaH_2PO_4 (pH 7.4, 22°C, 10 mmol/l), EDTA (5 mmol/l), sucrose (250 mmol/l). The homogenates were centrifuged at 200000 × *g* for 50 min and 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, pH 7.4, 22°C) and the protease inhibitors aprotinin (600 TIU/l), pepstatin (2 μmol/l), leupeptin (2 μmol/l) and PMSF (2 mmol/l). Insoluble material was removed by centrifugation at 200000 × *g* for 50 min. The clear supernatant was applied to columns (1 × 2.5 cm) of wheat germ agglutinin coupled to agarose with addition of 150 mmol/l NaCl and the same volume of Hepes buffer (25 mmol/l, pH 7.4, 22°C). The column was washed with 30–40 ml of a buffer containing 25 mmol/l Hepes, and 0.1% (by vol.) Triton X-100 and then the bound material was eluted with 10 ml of the washing buffer supplemented with 0.3 mol/l *N*-acetylglucosamine (flow rate 50–60 ml/h). 0.5-ml fractions of the eluate were collected, protein content and ^{125}I binding were determined as described [9] and the peak fractions were used for further studies. For the standard phosphorylation assay approx. 1 μg wheat germ purified protein was preincubated at 22°C for 30 min with insulin in different concentrations (10^{-10}–10^{-7} mol/l) or without insulin followed by an incubation with [^{32}P]ATP (5 or 40 μmol/l) in elution buffer containing 10 mmol/l $MnCl_2$ and 1 mmol/l vanadate at 22°C for 15 min. The incubation was stopped by addition of Laemmli buffer 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 out from the gel and counted in a scintillation counter.

3. RESULTS

Muscle of 5 patients (2–5 g wet tissue each) were obtained and the wheat germ purification procedure was performed as described in section 2. Three preparations yielded a sufficient amount of receptor kinase activity and these preparations were used for further studies. When the proteins partially purified by the wheat germ affinity pro-

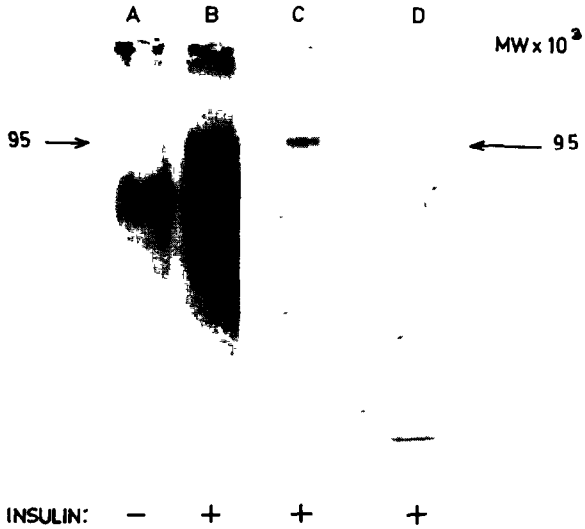

Fig.1. Autoradiogram of the phosphorylation of the insulin receptor from human skeletal muscle. Wheat germ affinity purified receptor from human skeletal muscle was incubated for 30 min at room temperature with insulin (10^{-8} mol/l). Phosphorylation was studied at 22°C with [^{32}P]ATP (40 μmol, 0.01 mCi) for 15 min. 1 μg protein was used in each assay. The reaction was stopped by boiling in Laemmli buffer. Phosphoproteins were analysed by SDS 10% 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 and D, 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 after stimulation of phosphorylation with insulin. Lane D shows the supernatant of the immunoprecipitate of insulin-stimulated phosphoproteins. Immunoprecipitates and supernatant were analyzed by SDS 10% polyacrylamide gel electrophoresis.

cedure were incubated with insulin for 30 min followed by a 15 min phosphorylation period, increased ^{32}P incorporation was seen in a 95-kDa band (fig.1, lanes A,B). If the phosphorylated proteins were immunoprecipitated with a serum containing insulin receptor antibody, the 95-kDa protein was quantitatively precipitated (fig.1, lane C) while no precipitation was seen with control serum (not shown), and no phosphorylation was seen in the supernatant of the immunoprecipitate (fig.1, lane D). This identifies the phosphorylated 95-kDa protein as the β-subunit of the insulin receptor.

The stimulation of receptor phosphorylation by insulin can also be demonstrated at insulin concentrations in the physiological range. Fig.2 shows the dose-response curve of insulin stimulation of phosphorylation of muscle receptor. At 10^{-9} mol/l insulin a half-maximal effect is reached approximately.

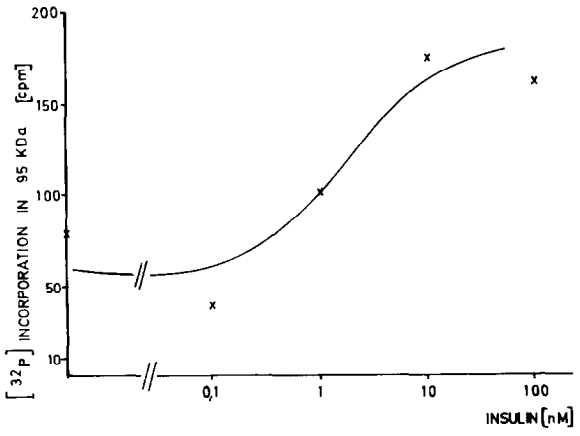

Fig.2. Dependence of insulin receptor phosphorylation on insulin concentration. Dose-response curve of insulin action on phosphorylation of the 95-kDa subunit of the insulin receptor. Wheat germ affinity purified receptor from skeletal muscle was incubated for 30 min at room temperature with insulin (10^{-10}–10^{-7} mol/l). Phosphorylation was studied at 22°C with [γ-^{32}P]ATP (40 μmol, 0.01 μCi) for 15 min. 1 μg protein was used in each assay. The reaction was stopped by boiling in Laemmli buffer. Phosphoproteins were analysed by SDS 10% polyacrylamide gel electrophoresis. The 95-kDa band was identified by autoradiography and the band was cut out from the gel and counted in a scintillation counter. A control band of equal size was counted to determine background activity and the value was subtracted from the value found in the 95-kDa band.

4. DISCUSSION

Human skeletal muscle contains receptor kinase which is activated by physiological insulin concentrations. Activation of insulin receptor kinase and autophosphorylation of the receptor had been intensively characterized in animal tissues, human non-target tissues and cell culture [1–10]. Kinetic properties, specificity and insulin sensitivity of receptor phosphorylation in rat liver cells [4], rat hepatoma cells [2,8,9], rat fat cells [3] and rat muscle [10] support the idea that this is the first signal transmitting step which follows insulin binding. The present findings in human skeletal muscle would be compatible with this model of insulin action. If the results obtained here with the human muscle are compared with the characteristics found in rat skeletal muscle [10] it is evident that the stimulation of receptor phosphorylation in the human tissue is much lower, 2-fold, compared to a 10–16-fold stimulation in the rat. However, this parallels characteristics of other insulin effects such as the stimulation of glucose transport in fat, where a 7–10-fold stimulation is found in the rat tissue [15], whereas the stimulation in the human tissue is only 2–3-fold [16].

The second purpose of this study was to determine how receptor kinase activity can be studied in muscle of type II diabetic patients. Based on glucose clamp studies in type II diabetics [11] it was suggested that insulin resistance in these patients is predominantly caused by an impaired insulin effect in skeletal muscle. On the other hand, several studies have appeared now [12–14] which showed that decreased receptor autophosphorylation is found in several situations with insulin resistance. The study of receptor autophosphorylation in muscle of type II diabetic patients should clarify if an abnormality of receptor kinase contributes to the insulin resistance in these patients. Based on the present results the small amounts of muscle tissue which can be obtained by biopsy [17] and which were used to study insulin binding are not yet sufficient to study receptor kinase. A substrate assay of the kinase might solve this problem and this question is currently under investigation.

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