Mechanism of insulin receptor kinase inhibition in non-insulin-dependent diabetes mellitus patients. Phosphorylation of serine 1327 or threonine 1348 is unaltered.

M Kellerer, … , K Siddle, H U Häring

Research Article

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Mechanism of Insulin Receptor Kinase Inhibition in Non–Insulin-dependent Diabetes Mellitus Patients

Phosphorylation of Serine 1327 or Threonine 1348 Is Unaltered

Monika Kellerer,* Matthew Coghlan,‡ Edison Capp,* Andreas Mühlhöfer,* Gerhild Kroder,* Luitgart Mosthaf,*
Paola Galante,* Kenneth Siddle,‡ and Hans Ulrich Häring*

*Institut für Diabetesforschung, Kölner Platz 1, D-80804 München, Germany; and ‡Cambridge University, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge CB2 2QR, United Kingdom

Abstract

The tyrosine kinase activity of insulin receptor isolated from the skeletal muscle of NIDDM patients has previously been found to be decreased compared with the activity of receptor from nondiabetic subjects but the mechanism underlying this defect is unknown. Phosphorylation of receptor serine/threonine residues has been proposed to exert an inhibitory influence on receptor tyrosine kinase activity and Ser 1327 and Thr 1348 have been identified as specific sites of phosphorylation in the insulin receptor COOH terminal domain. To address the potential negative regulatory role of phosphorylation of these residues in vivo, we assessed the extent of phosphorylation of each site in insulin receptor isolated from the skeletal muscle of 12 NIDDM patients and 13 nondiabetic, control subjects. Phosphorylation of Ser 1327 and Thr 1348 was determined using antibodies that specifically recognize insulin receptor phosphorylated at these sites. In addition, a phosphotyrosine-specific antibody was used to monitor receptor tyrosine phosphorylation. The extent of insulin-induced tyrosine autophosphorylation was decreased in receptor isolated from diabetic versus nondiabetic muscle, thus confirming earlier reports. In contrast, there was no significant difference in the extent of phosphorylation of either Ser 1327 or Thr 1348 in receptor isolated from diabetic or nondiabetic muscle as assessed by immunoprecipitation (Ser 1327: $5.6\pm1.6\%$ diabetics vs. $4.7\pm2.0\%$ control; Thr 1348: $3.8\pm1.0\%$ diabetics vs. $3.2\pm1.2\%$ control). Moreover, within each group there was no correlation between the level of tyrosine kinase activity and the extent of serine/threonine phosphorylation. It is concluded that the stoichiometry of serine/threonine phosphorylation of insulin receptor in vivo is low, and that increased phosphorylation of Ser 1327 or Thr 1348 is not responsible for the decreased insulin receptor tyrosine kinase activity observed in the skeletal muscle of NIDDM patients. (*J. Clin. Invest.* 1995. 96:6–11.) Key words: NIDDM • serine/threonine phosphorylation • insulin receptor • insulin resistance

Address correspondence to Hans Ulrich Häring, Institut für Diabetesforschung, Kölner Platz 1, D-80804 München, Germany. Phone: 89-307-93121; FAX: 89-308-1733.

Introduction

Non–insulin-dependent diabetes mellitus (NIDDM)[1] is characterized by abnormalities of insulin secretion and by insulin resistance of the major target tissues (1). In particular, insulin resistance of the skeletal muscle appears to play a pivotal role in the pathogenesis of the disease (2). Several studies have previously investigated whether an impaired signalling capacity of the insulin receptor contributes to the pathogenesis of skeletal muscle insulin resistance. Most studies have reported that autophosphorylation or substrate phosphorylation of the insulin receptor tyrosine kinase isolated from diabetic skeletal muscle are reduced (3–9). The molecular mechanisms responsible for the reduced activation of the insulin receptor kinase in NIDDM patients have not yet been defined. However, the defect appears to be acquired rather than inherited as insulin receptor mutations are extremely rare in common forms of NIDDM (10).

Conditions modulating the signalling function of the insulin receptor kinase have been studied in many different cell systems. It has been shown that factors such as hyperinsulinemia, hypoinsulinemia, and hyperglycemia, as well as agonists including catecholamines, adenosine, and phorbol esters, are able to regulate insulin receptor function (reviewed in reference 11). In addition, evidence for a potential regulatory role of G-proteins in insulin receptor function has been reported (12). However, studies with phorbol esters (13), hyperglycaemic conditions (14), and catecholamines (15), are of particular importance as they suggest that activation of protein serine/threonine kinases, that are able to phosphorylate the insulin receptor, might be involved in receptor kinase inhibition. It has been speculated that an analogous mechanism might be responsible for the reduced insulin receptor kinase activity observed in NIDDM patients (16–18). There is, however, no evidence to date in support of this hypothesis as it has not been possible to determine the serine and threonine phosphorylation state of receptor in vivo.

Recently, several groups have succeeded in identifying serine and threonine phosphorylation sites within the insulin receptor COOH-terminal region which might be important for modulation of receptor kinase activity (19–23). Antibodies have been raised against phosphopeptides corresponding to the sequences surrounding two of these sites (Ser 1327 and Thr 1348). These antibodies (anti-PS1327, anti-PT1348) react specifically with insulin receptor phosphorylated at Ser 1327 or Thr

1348 (23). Therefore, anti-PS1327 and anti-PT1348 antibodies enable the phosphorylation state of two sites potentially involved in the regulation of insulin receptor function to be determined. In the present study these antibodies have allowed us, for the first time, to investigate whether the impaired kinase activity of insulin receptor, isolated from NIDDM patients, is associated with increased receptor serine/threonine phosphorylation.

Methods

Materials. Human recombinant insulin was purchased from Eli Lilly (Indianapolis, IN). Phorbol ester TPA was from Sigma (Munich, Germany). Polyclonal anti-phosphotyrosine antibody was purchased from Gibco (Eggenstein, Germany). ^{125}I-Insulin was from NEN DuPont (Bad Homburg, Germany). All gel electorphoresis equipment, HRP-conjugated anti−rabbit JgG, and protein standards for SDS-gel electrophoresis were from BioRad (Munich, Germany). All other reagents were of the best grade commercially available. Rat-1 fibroblasts stably overexpressing the human insulin receptor isoform B (exon +11) were kindly provided by A. Ullrich (Max-Planck-Institut, Martinsried, Germany).

Characterization of tissue samples. Tissue specimens of musculus gastrocnemius were obtained from non-obese Type II diabetic and non-diabetic 60–80-yr-old subjects who had undergone leg amputation due to peripheral arteriovascular complications. Informed consent was obtained from all the subjects. All samples were taken from the musculus gastrocnemius immediately after amputation, cut into small pieces, rapidly frozen in liquid nitrogen and stored at −80°C.

Methods for histological, histochemical, and morphological examination as well as for measuring muscle enzyme activities have been previously described in detail (7).

Partial purification of insulin receptors from skeletal muscle through wheat germ agglutinin–chromatography. The skeletal muscle samples (4 grams of tissue) were homogenized by an ultra turrax for 10–15 s at maximal speed at 4°C in the presence of protease inhibitors PMSF (2 mM), aprotinin (1,200 trypsin inhibiting units), benzamidine (10 mM), bacitracin (7,500 U/l) and leucine (10 mM) in a 25 mM Hepes buffer containing $Na_4P_2O_7$ (10 mM), NaF (100 mM) and sodium-orthovanadate (1 mM). The lysate was centrifuged for 20 min at 20,000 *g* and 4°C. The supernatant was discarded. The pellet was solubilized in the same Hepes buffer as above but supplemented with 1% Triton X-100 and then centrifuged for 50 min at 200,000 *g* to remove insoluble material. The supernatant was then applied to a column of wheat germ agglutinin (WGA) coupled to agarose. After extensive washing with 25 mM Hepes buffer containing protease and phosphatase inhibitors as described above and supplemented with 0.05% Triton X-100 the bound material was eluted with 0.3 M *N*-acethylglucosamine dissolved in the same buffer.

Immunoblot with anti-phosphotyrosine and anti-phosphopeptide antibodies. Rat-1 fibroblasts stably overexpressing the human insulin receptor isoform B (HIR-B, exon +11) were incubated for 20 min with or without 100 nM TPA. The supernatant was discarded and cells were lysed on ice with 25 mM Hepes buffer containing 1% Triton X-100, 1 mM PMSF, 1200 TIU/1 aprotinin, 1 mM sodium orthovanadate, 10 mM $Na_4P_2O_7$, 100 mM NaF. Samples were boiled for 5 min in Laemmli sample buffer (reducing) and resolved by electrophoresis on SDS-poly-acrylamide gels (7.5%). Proteins were transferred to nitrocellulose by electroblotting (transfer buffer: 20 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH 8.8). After transfer, the filters were blocked with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton X-100, 0.25% gelatine, pH 7.4) for 1 h. Subsequently they were incubated with the anti-phosphoserine (anti-PS1327) and anti-phosphothreonine (anti-PT1348) antibody for 16 h at 4°C. The nitrocellulose membranes were washed 4 × 10 min with NET buffer before incubating with horseradish peroxidase−conjugated anti−rabbit IgG for 1 h at 21°C. Visualization of immunocomplexes was performed by enhanced chemiluminescence (ECL, Amersham, Germany).

Dephosphorylation of serine(P) and threonine(P)-containing insulin receptor with alkaline phosphatase. Immobilized alkaline phosphatase was prepared as recommended by BioRad and as described (24). Briefly, 5 mg (1,000 U/mg) of alkaline phosphatase from bovine intestinal mucosa (Sigma) was coupled to 0.5 ml of Affi-gel 10 (BioRad) in 100 mM Hepes buffer. The immobilized alkaline phosphatase was washed with 50 mM Hepes containing 0.1% Triton X100. 3 μg of WGA-purified receptor was incubated with Affi-gel 10 coupled with or without alkaline phosphatase for 2 h at 4°C. The supernatant was separated from the Affi-gel 10 and boiled with Laemmli buffer for 5 min. 7.5% Polyacrylamide SDS-gel electrophoresis and immunoblotting with the anti-phosphopeptide antibodies (anti-PS1327, anti-PT1348) was performed as described above.

Autophosphorylation of the partially purified insulin receptor. 3 μg of total protein eluted from the wheat germ agglutinin agarose column was preincubated for 30 min with or without 100 nM insulin. This was followed by an incubation with 5 μM ATP for 10 min. The phosphorylation reaction was stopped by addition of Laemmli buffer and by boiling at 95°C for 5 min. 7.5% SDS-PAGE and western blotting were performed as described above. Phosphorylation of the insulin receptor was assessed using an anti-phosphotyrosine antibody.

Co-precipitation of receptor/^{125}I-insulin complexes. For quantification of phosphorylation of the insulin receptor COOH terminus, at residues Ser 1327 and Thr 1348 co-precipitation of receptor/^{125}I-insulin complexes was performed as described (23). Briefly, partially purified receptor from human skeletal muscle was preincubated for 16 h at 4°C with ^{125}I-insulin (40,000 cpm) in a buffer containing 50 mM Tris/HCl, 1% BSA, 10 mM $MgSO_4$, pH 7.8. The insulin receptor was precipitated using antibody anti-PS1327, anti-PT1348 (both at dilutions of 1:500) or the anti-insulin receptor monoclonal antibody 83-14 (dilution 1:1000) (25) in the presence of protein A−sepharose, for 2 h at 4°C. Samples were then washed three times in ice cold wash buffer containing 25 mM Hepes, 0.01% Triton X-100, 1 mM PMSF, and 100 mM NaF at pH 7.5. The ^{125}I-insulin labeled insulin receptor immunocomplexes were counted in a gamma counter.

Results

12 NIDDM patients and 13 nondiabetic controls were studied. Table I shows the clinical data of the two groups. Tissue samples of NIDDM patients and non-diabetic controls were characterized as described previously (7). No significant differences with respect to fiber composition, fiber size, or ischemia sensitive enzymes were found. To investigate whether polyclonal anti-phosphopeptide antibodies which have been described recently (23) are able to detect specific phosphorylation sites of the human insulin receptor COOH terminus we used these antibodies to immunoblot extracts of rat-1 fibroblasts stably overexpressing the human insulin receptor isoform B (HIR-B, exon +11) that had been treated with or without 100 nM TPA. An increased signal was detected with both site specific antibodies after TPA treatment of rat-1 fibroblasts (Fig. 1 *A*). This is in agreement with earlier reports showing that Ser 1327 and Thr 1348 of the insulin receptor are TPA induced phosphorylation sites (22, 23, 26).

The efficiencies of both phospho-peptide antibodies to immunoprecipitate insulin receptors phosphorylated at the respective site after TPA treatment were determined with rat1-HIR-B-fibroblasts. Fig. 1 *B* shows a western blot of an immunoprecipitation of insulin receptors and the supernatant with the anti-PS 1327 and the anti-PT 1348 antibody. Both antibodies immunoprecipitate under these conditions > 90% of Ser 1327 or Thr 1348 phosphorylated receptors. The amount of insulin receptors isolated from skeletal muscle used in the further immunoprecipitation experiments were far below the capacity of these antibod-

Table I. Characteristics of the Subjects Studied

| | n | Age | Fasting serum | | Hb/A$_{1c}$ | Insulin-treated | Oral antidiabetics |
			Glucose	Insulin			
		yr	mmol/l	μU/ml	%		
Nondiabetic	13	73.7±10.7	5.0±0.5	9.0±4.7	5.6±0.4	None	None
Diabetic	12	72.6±9.8	11.2±3.5	14.3±8.5	7.9±2.3	9	4

ies providing conditions where immunoprecipitation is almost quantitative (> 90%).

As a further control to test whether this antibody recognizes the phosphorylated state of the insulin receptor, experiments with alkaline phosphatase were performed. Partially purified insulin receptor from human skeletal muscle was incubated with or without immobilized alkaline phosphatase as described in Methods. Fig. 1 C shows that both antibodies recognize insulin receptors from human skeletal muscle. However, if these receptors are first treated with alkaline phosphatase the signal disappears. This result demonstrates that the antibodies recognize only the phosphorylated forms of Ser 1327 and Thr 1348 of the insulin receptor and is in agreement with earlier studies demonstrating that this antibody does not react with an insulin receptor mutated at Ser 1327 (M. Coghlan, unpublished observation).

To investigate whether the decreased insulin receptor tyrosine kinase activity in skeletal muscle of NIDDM patients is due to increased serine/threonine phosphorylation of the receptor COOH terminus we compared the extent of phosphorylation of Ser 1327 and Thr 1348 in diabetic and non diabetic muscle. Co-immunoprecipitation experiments with each antibody were performed and compared with immunoprecipitation of total receptor as determined using a monoclonal insulin receptor antibody (83-14). The latter had previously been shown to react with receptors independently of its state of phosphorylation (M. Coghlan, unpublished observation). The percentage of receptors immunoprecipitated with the anti-PT1348 and the anti-PS1327 antibody are indicated in Fig. 2. Fig. 2 (left) shows that there is no significant difference between NIDDM patients and nondiabetic controls in the mean values and the distribution of the phosphorylation of Thr 1348 detected with the site-specific antibody anti-PT1348. Comparable results were obtained with the site specific serine antibody anti-PS1327 (Fig. 2, right). Immunoprecipitation of insulin receptors with the anti-PS1327 antibody revealed no significant difference in the mean value between the NIDDM and control group. However, a slight tendency for an increase in serine phosphorylation is seen in some NIDDM patients.

Even though there is no difference in the mean values of the COOH-terminal serine/threonine phosphorylation between the NIDDM and control group, it could be possible that individual patients with a comparably high state of phosphorylation of Ser 1327 and Thr 1348 show especially low insulin receptor kinase activity. To prove this hypothesis we analyzed whether individual values of COOH-terminal serine/threonine phosphorylation correlate with insulin stimulated insulin receptor autophosphorylation. The insulin receptor kinase activity of control and NIDDM patients was measured in vitro by immunoblotting receptor with an anti-phosphotyrosine antibody. We

selected four NIDDM patients with particularly low kinase activity compared with the control group. The immunoblots with the anti-phosphotyrosine antibody from 4 NIDDM and 4 control subjects are shown in Fig. 3. We then investigated whether we could detect any differences in the serine/threonine phosphorylation of the insulin receptor COOH terminus between the group with the high (4 control subjects) and that with the low (4 NIDDM patients) tyrosine kinase activity. Fig. 4 shows that no tendency for increased phosphorylation of Ser 1327 or Thr 1348 was detectable in receptors with reduced kinase activity.

Discussion

Skeletal muscle insulin resistance plays a central role in the development of NIDDM. As outlined in the introduction and as reviewed recently in more detail (1, 11) it is unlikely that a primary defect at the receptor level exists. However, negative modulation of the insulin receptor kinase is most likely an important factor contributing to overall insulin resistance of the skeletal muscle in NIDDM (3–9). There is evidence that insulin resistance of the skeletal muscle in a given individual is determined by multiple factors and that it may be partially reversible under certain conditions (27). Several clinical studies applying the glucose clamp technique have shown that the insulin resistance of the skeletal muscle depends on factors such as exercise (28–30), weight reducing dietary regimen (1, 31, 32), metabolic control and drug treatment (1, 2, 33). Better metabolic control, i.e., lowering of glucose levels, muscle exercise, and reduction of body fat, in particular when central obesity exists, seem to improve the insulin sensitivity of the skeletal muscle (28–33). Data from isolated cell systems suggest that modulation of the insulin receptor function may be important in this context (27). The conflicting results obtained by different groups studying the activity of insulin receptor isolated from the skeletal muscle of NIDDM patients most likely reflect variation in the modulatory situation of the receptors in these patients.

In our study group all patients were rather old, all NIDDM patients had a long history of diabetes and were at least before surgery under insulin treatment. Furthermore, all individuals were at bedrest several days before surgery. In both groups the majority of patients were taking antibiotics and other medication. Even though we have tried to match muscle samples as described in our earlier publications (7) as carefully as possible, influences of the specific clinical situation cannot be excluded.

Modulation of the insulin receptor function in different cell models was demonstrated for a number of different agents. Studies with phorbol esters which activate protein kinase C, gave a first insight into the potential mechanisms underlying insulin receptor modulation (34). Even though different groups
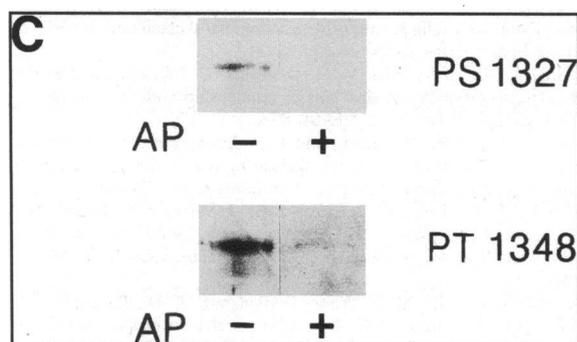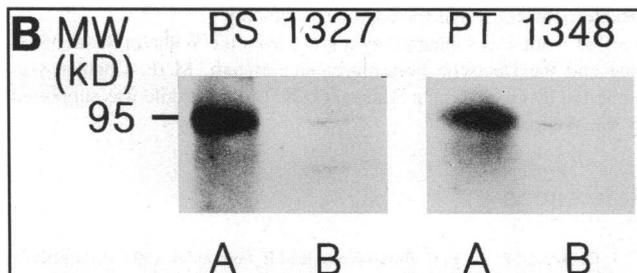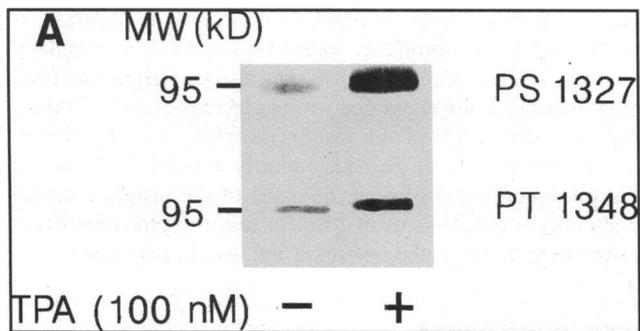
Figure 1. (A) Immunoblot of human insulin receptor (HIR-B) with antibodies against phosphoserine 1327 and phosphothreonine 1348. Rat-1 fibroblasts stably overexpressing the human insulin receptor isoform B (HIR-B, +exon 11) were treated without (−) or with (+) the phorbol ester TPA (100 nM) for 20 min. Subsequently cells were lysed in the presence of phosphatase and protease inhibitors and proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose and immunoblots were performed as described in Methods with the anti-PS1327 or anti-PT1348 antibody. (B) Immunoprecipitation of insulin receptors from phorbol ester TPA-treated cells. Rat-1 fibroblasts overexpressing the human insulin receptor isoform B were incubated 20 min with 100 nM phorbol ester (TPA). Confluent cells of 1 culture plate (96 cm²) were lysed in 1.2 ml Hepes buffer containing protease and phosphatase inhibitors and 60 μl of this lysate were immunoprecipitated either with anti-PS 1327 (dilution 1:500) or anti-PT 1348 (dilution 1:500) antibodies. Immunoprecipitates (lanes A) and the supernatant (lanes B) were applied to a 7.5% SDS-PAGE and immunoblots were performed with the same antibodies used for the immunoprecipitation (anti-PS 1327 or anti-PT 1348). (C) Immunoblot of WGA-purified insulin receptor from human skeletal muscle. Insulin receptors were partially purified from human skeletal muscle as described in Methods. The partially purified insulin receptors were dephosphorylated by immobilized alkaline phosphatase as described in Methods. Receptors were separated by SDS-PAGE and immunoblotted with anti-PS1327 or anti-PT1348 antibody. The first lanes show immunoblots with untreated receptors (−), the second lanes show immunoblots from insulin receptors which were treated with alkaline phosphatase (AP) in vitro (+).
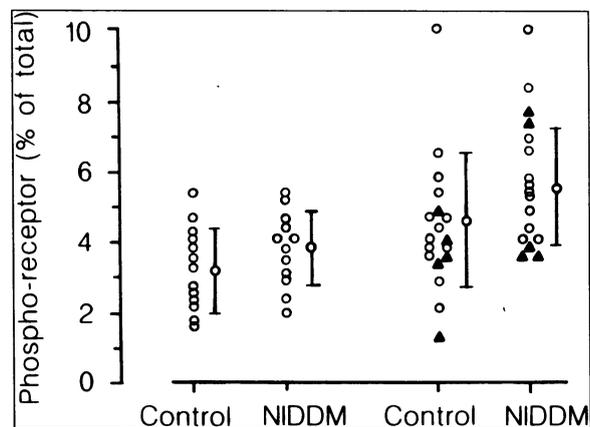

Figure 2. Determination of Thr 1348 and Ser 1327 phosphorylated insulin receptors from skeletal muscle of NIDDM patients and non-diabetic subjects. WGA-purified insulin receptor was pre-incubated with ¹²⁵I-insulin, and the resulting receptor/¹²⁵I-insulin complex precipitated using antibody, anti-PT1348 (left) and anti-PS1327 (right) as described in Methods. Results are expressed as the percentage of total receptor, as determined by precipitation with the anti-insulin receptor monoclonal antibody, 83-14. (▲) samples assayed in Cambridge.

have obtained different results in phorbol ester-treated cells (13, 14, 34–36), it seems justified to conclude that at least in certain cell types activation of protein kinase C results in serine phosphorylation of the insulin receptor (14, 23, 26, 35). This serine phosphorylation is associated with decreased receptor activity (14, 35). Hyperglycemia-induced inhibition of receptor kinase activity is also believed to be caused by increased PKC-mediated receptor serine/threonine phosphorylation (14). It is at present unclear which PKC isoforms are involved in this effect. Furthermore, it is unclear where the regulatory sites in the receptor are located. However, it has been suggested that Thr 1348 is a phosphorylation site in the receptor COOH terminus which is phosphorylated by protein kinase C after phorbol ester stimulation (22, 23). Earlier studies in CHO-cells suggested that after phorbol ester stimulation ~ 25% of receptors were phosphorylated at Thr 1348 (23). Recently phorbol ester stimulated insulin receptor phosphorylation was demonstrated for another potential regulatory site, Ser 1327 (23, 26). The two anti-phosphopeptide antibodies used in the present study are able to
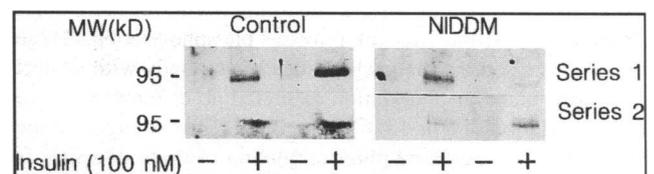

Figure 3. Autophosphorylation of the insulin receptor β-subunit from NIDDM patients and control subjects. Insulin receptor from skeletal muscle was partially purified by WGA and phosphorylated in vitro without (−) or with (+) 100 nM insulin as described in Methods. Proteins were separated by 7.5% PAGE and electrotransferred to nitrocelluloses as described in Methods. Phosphorylation of the insulin receptor was measured by immunoblotting with an polyclonal antiphosphotyrosine antibody and visualized with using chemiluminiscense (ECL, Amersham). Autophosphorylation of insulin receptors from four control subjects and from four NIDDM patients is shown in the presence (+) or absence (−) of 100 nM insulin.
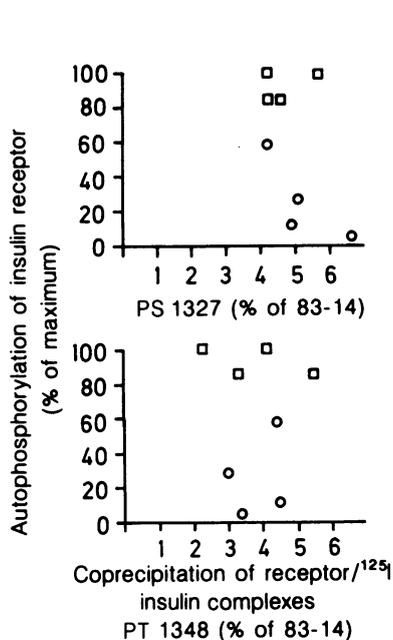
Figure 4. Correlation of insulin receptor autophosphorylation with the state of receptor serine/threonine phosphorylation. The insulin induced (100 nM insulin) state of autophosphorylation of receptor isolated from four NIDDM (○) and four control subjects (□), is plotted as the Y coordinate. Maximum autophosphorylation is given as 100%. The extend of receptor phosphorylation at Ser 1327 and Thr 1348, is plotted as the X co-ordinate. No correlation exists between phosphorylation of these sites and the state of receptor autophosphorylation.

detect increased phosphorylation of these two sites (Ser1327 and Thr1348) in receptor from phorbol ester-stimulated cells (23). With both antibodies a clear signal is obtained in insulin receptor from patients, which is reversed after alkaline phosphatase treatment. This experiment shows that the signal obtained with these antibodies does indeed reflect the phosphorylation status of the receptors. The binding data after immunoprecipitation of receptors with these two antibodies show that $\sim$ 2–8% of the receptors are phosphorylated at these two potential regulatory sites. However, the observation that there is no significant difference in the mean values of Thr 1348 phosphorylation between diabetic and control receptor suggests that this site is not involved in the regulation of tyrosine kinase activity in the skeletal muscle of NIDDM patients. As shown in earlier studies (4, 7) there is a high individual variation of the insulin stimulated kinase activity in NIDDM patients and controls. As this variation might prevent the efficient detection of a correlation between mean values of increased serine or threonine phosphorylation with reduced kinase activity we have selected a subgroup of the NIDDM patients which show a blunted insulin effect on tyrosine kinase activation. We have compared the results in this subgroup with a subgroup of control individuals showing high stimulation of tyrosine phosphorylation. These two groups of our patients which differ maximally with respect to tyrosine autophosphorylation exhibited no differences in the phosphorylation of Thr 1348 or Ser 1327. This strengthens the conclusion that increased phosphorylation of these two sites is probably not causally related to the reduced tyrosine kinase activity of receptors from NIDDM patients. However, our results do not exclude the possibility that PKC is involved in the pathogenesis of insulin resistance in skeletal muscle. It is conceivable that other regulatory sites in the insulin receptor β subunit are important for the suppression of the insulin receptor kinase activity. It was shown earlier that phorbol esters are able to inhibit the tyrosine kinase activity of COOH-terminally truncated insulin receptors demonstrating that PKC-mediated insulin receptor kinase inhibition might not require phosphorylation of COOH-terminal serine or threonine residues (37). We

ourselves have recently obtained similar results in hyperglycemia-treated rat-1 fibroblasts expressing a COOH-terminally truncated insulin receptor (26). It was also suggested that PKC might induce insulin resistance without increasing serine/threonine phosphorylation of the insulin receptor (37, 38). Therefore, further studies are needed to clarify whether PKC-dependent phosphorylation of other domains of the insulin receptor or alternative mechanisms of PKC/insulin receptor interaction are involved in the pathogenesis of the insulin resistance.

Acknowledgments

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