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A Defective Intramolecular Autoactivation Cascade May Cause the Reduced Kinase Activity of the Skeletal Muscle Insulin Receptor from Patients with Non-insulin-dependent Diabetes Mellitus*

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The insulin receptor purified from skeletal muscle of patients with non-insulin-dependent diabetes mellitus (NIDDM) displayed a 25–55% reduction in insulin-stimulated autophosphorylation and tyrosyl-specific phosphotransferase activity relative to controls. This decrease was not explained by alterations of muscle fiber composition, insulin binding affinity or capacity, or the K_m values for ATP; the lower kinase activity was entirely attributed to a decrease in the V_{max} of the enzyme. Phosphorylation sites in the β-subunit of the control and diabetic receptor were identified by tryptic digestion and reverse-phase high performance liquid chromatography. Autophosphorylation occurred primarily in two regions of the β-subunit: the regulatory region containing Tyr-1146, Tyr-1150, and Tyr-1151, and the C terminus containing Tyr-1316 and 1322. Autophosphorylation of the regulatory region at all three tyrosyl residues (tris-phosphorylation) appears to be necessary to activate the receptor kinase (White, M. F., Shoelson, S. E., Stepman, E. W., Keutmann, H. & Kahn, C. R. (1988) *J. Biol. Chem.* 263, 2969–2980). The receptor from NIDDM patients showed a decreased level of tris-phosphorylation of the regulatory region which was closely associated (r^2 = 0.97) with the decreased kinase activity. In contrast, weak associations were found between kinase activity and the bis-phosphorylated forms of the regulatory region (r^2 = 0.51) and the C terminus (r^2 = 0.35). Therefore, the reduced formation of the tris-phosphorylated regulatory region in the diabetic receptors suggests that a defective autophosphorylation cascade leading to tris-phosphorylation of the regulatory region may cause, in part, the reduced insulin-stimulated kinase activity of the insulin receptor in muscle of NIDDM patients.

Non-insulin-dependent diabetes mellitus (NIDDM)[1] is characterized by abnormalities of insulin secretion and by insulin resistance in target tissues such as muscle and fat (1–13). Since skeletal muscle plays the predominant role in post-prandial glucose disposal, insulin resistance in this tissue represents an essential factor in the pathogenesis of NIDDM (1–14). It is not known which step of the transmembrane signaling mechanism of the insulin receptor is defective in NIDDM patients.

Transmission of the insulin signal across the plasma membrane of target cells presumably occurs through activation of the intrinsic tyrosine kinase in the β-subunit of the insulin receptor (14–21). This kinase undergoes insulin-stimulated autophosphorylation, which increases phosphorylation of other cellular substrates by the receptor (22–29). A phosphorylation cascade may amplify the insulin signal to activate the metabolic machinery of the target cell (22). A defective insulin receptor kinase was found in several experimental models of insulin resistance, and it was speculated that this defect causes the insulin-resistant state (for review see Ref. 30). *In vitro*, reduced insulin receptor kinase activity was recently found in peripheral tissues of patients with NIDDM, especially in the skeletal muscle (31–34). However, the mechanism leading to the reduced activity of the receptor kinase in skeletal muscle of NIDDM patients is not understood. A change of the fiber composition of the skeletal muscle due to NIDDM may cause the altered kinase activity, as the different fiber types (type I or type II) contain different kinase activities (35–37). Alternatively, activation of the receptor kinase by insulin binding may be impaired.

In this study, we analyzed the fiber composition of muscle probes from non-diabetic and NIDDM patients. We found that the skeletal muscle composition of all the samples was identical, which suggested that a defect intrinsic to the kinase itself exists in NIDDM. To analyze the molecular basis of this defect, we isolated and characterized the insulin receptor kinase from skeletal muscle of non-obese NIDDM patients and non-obese controls. Our results suggest that an impaired autophosphorylation cascade, especially in the regulatory region of the β-subunit which contains three tyrosyl phosphorylation sites (Tyr-1146, 1150, and 1151), may be responsible for the reduced activation of the kinase.

EXPERIMENTAL PROCEDURES[2]

RESULTS

The Selection of Skeletal Muscle Biopsies—Muscle samples from non-diabetic or NIDDM patients were characterized by

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[1] The abbreviations used are: NIDDM, non-insulin-dependent diabetes mellitus: HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 2–5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE I

Patient characteristics

The characteristics of the patient population. The fasting glucose levels for each patient are mean values of three to four morning glucose measurements during clinical treatment; fasting insulin levels were obtained from one serum probe taken in the morning before breakfast, 2–3 weeks after the amputation when the patients had been mobilized (means ± S.D.)

Patient group	Muscle type	No.	Age	Body mass index	Fasting serum	
					Glucose	Insulin
			years	*kg/m²*	*mg/dl*	*microunits/ml*
I	Non-diabetic gastrocnemius	8	69 ± 9	20 ± 2	79 ± 7	11 ± 1
II	NIDDM gastrocnemius	9	73 ± 9	20 ± 2	200 ± 51	25 ± 2
III	Non-diabetic latissimus dorsi (3 preparations)	12	46 ± 6	22 ± 3	82 ± 12	10 ± 2

morphological and biochemical criteria (Table I). First, the physiological integrity of the tissue was assessed by light microscopy. Samples which showed signs of ischemia were excluded and only normal tissue was further characterized. The selected skeletal muscle samples were placed into three groups: Group I was composed of eight muscle probes obtained from musculus gastrocnemius of non-diabetic patients undergoing leg amputations; group II was composed of nine gastrocnemius probes from NIDDM patients undergoing leg amputations; group III was composed of 12 small probes pooled into 3 sets from musculus latissimus dorsi of non-diabetic patients undergoing plastic reconstructive surgery. The third group was used as an additional non-diabetic control obtained by a different surgical technique and at a younger age.

The fiber composition of the selected muscle samples was determined histologically. Fig. 1*A* shows representative sections of a non-diabetic (*left*) and a NIDDM (*right*) gastrocnemius. Muscle fibers obtained from the non-diabetic and NIDDM patients were quantified by surface integration of sections like those shown in Fig. 1*A*. The distribution of fibers according to their square dimension was identical for both groups (*B*). The total square dimension of the two fiber types showed no significant differences between groups I and II (*C*). These data indicate that the muscle fiber composition of the control and NIDDM muscle was identical. Thus, altered fiber composition does not explain the reduced kinase activity of the receptor purified from muscle of NIDDM patients.

The activity of characteristic muscle enzymes was measured, and the amount of non-collagen protein was determined in each muscle sample (Table II). Phosphoglycerate kinase, phosphofructo kinase, lactate dehydrogenase, and phosphoglycero mutase were measured twice for every muscle sample. The enzyme activities of the tissue chosen for our study were not significantly different between non-diabetic and NIDDM muscle. Similarly, non-collagen protein was identical in all the samples. Therefore, only muscle tissue of comparable quality was used for analysis of the insulin receptor kinase.

Kinetic Parameters of the Insulin Receptor Kinase from Normal and NIDDM Patients—To characterize the receptor kinase defect, we measured the values for K_m and V_{max} for the autophosphorylation activity. During a 2-min incubation, a linear increase in the autophosphorylation activity for both patient groups was observed. However, autophosphorylation of the diabetic receptor was slower (Fig. 6*A*, *inset*). By Lineweaver-Burk analysis, no significant change in the K_m for ATP was detected, but we found a 2-fold reduction in the maximum velocity for autophosphorylation (V_{max}) in the diabetic group (*A*). The K_m for ATP and the V_{max} for poly(Glu:Tyr) phosphorylation was determined during a 60-min assay in the presence of 10^{-7} M insulin. There was no change of the K_m for ATP, whereas the average V_{max} for poly(Glu:Tyr) was reduced about 3-fold (*B*).

Tryptic Peptide Mapping—Phosphopeptides were separated from tryptic digests of the β-subunit obtained from six control patients (four from group I and two from group III) and five NIDDM patients. The peptide map for the six control receptor preparations showed identical HPLC-profiles with no significant variation in the elution times or the level of phosphorylation of the peptides (Fig. 7*A*). HPLC-profiles of the β-subunit from the NIDDM patients were qualitatively the same. However, each peptide was phosphorylated less during maximal insulin stimulation (Fig. 7, *B–E*).

Recently, the tryptic phosphopeptides separated by HPLC on a Bio-Rad RP-318 column were identified by a variety of techniques (51). In order to identify the peptides in our elution pattern using the LKB C_{18} column, we separated portions of three representative tryptic digests on the Bio-Rad RP-318 column as previously described (51) and on the LKB column used in this study. Similar patterns were obtained on each system (data not shown). Furthermore, by separation of single peaks from each column on the other, it was possible to identify each peak in our profile. The results are shown in Fig. 7 by using the numbering of White *et al.* (51). The first two peaks correspond to tris-phosphorylated peptide that contain Tyr(P)-1146, Tyr(P)-1150, and Tyr(P)-1151.[3] The fragments labeled pY4 and pY5 migrated as the corresponding bis-phosphorylated peptides that contain Tyr(P)-1146 and Tyr(P) at position 1150 or 1151. The remaining peaks (pY2 and pY3) contained the C-terminal phosphotyrosyl residues at 1316 and 1322.

Phosphorylation of the regulatory region and the C terminus of the β-subunit varied in samples from NIDDM patients, whereas their phosphorylation was nearly identical in all the samples from control patients. Moreover, the activity of the receptor measured with poly(Glu:Tyr) varied between the NIDDM patient samples, but it was identical in all of the controls. Therefore, we correlated the relative activity of the receptor to the extent of phosphorylation in the C terminus and in the regulatory region (Fig. 8). The relative kinase activity of the receptor from NIDDM patients was poorly associated ($r^2 = 0.41$) with total autophosphorylation (Fig. 8*A*). In contrast, a strong association ($r^2 = 0.97$) was found between kinase activity and tris-phosphorylation of the regulatory region (*B*). Kinase activity was poorly associated with bis-phosphorylation (pY4 + pY5) of the regulatory region ($r^2 = 0.51$) or phosphorylation (pY2 + pY3) of the C terminus ($r^2 = 0.35$) (*C* and *D*, respectively). Thus, tris-phosphorylation of the regulatory region of the β-subunit appears to be necessary to activate the kinase, whereas bis-phosphorylation of

[3] The numbering sequence used is this paper was described by Ullrich *et al.* (52) and is based on the position of the amino acids in the precursor of the human insulin receptor.

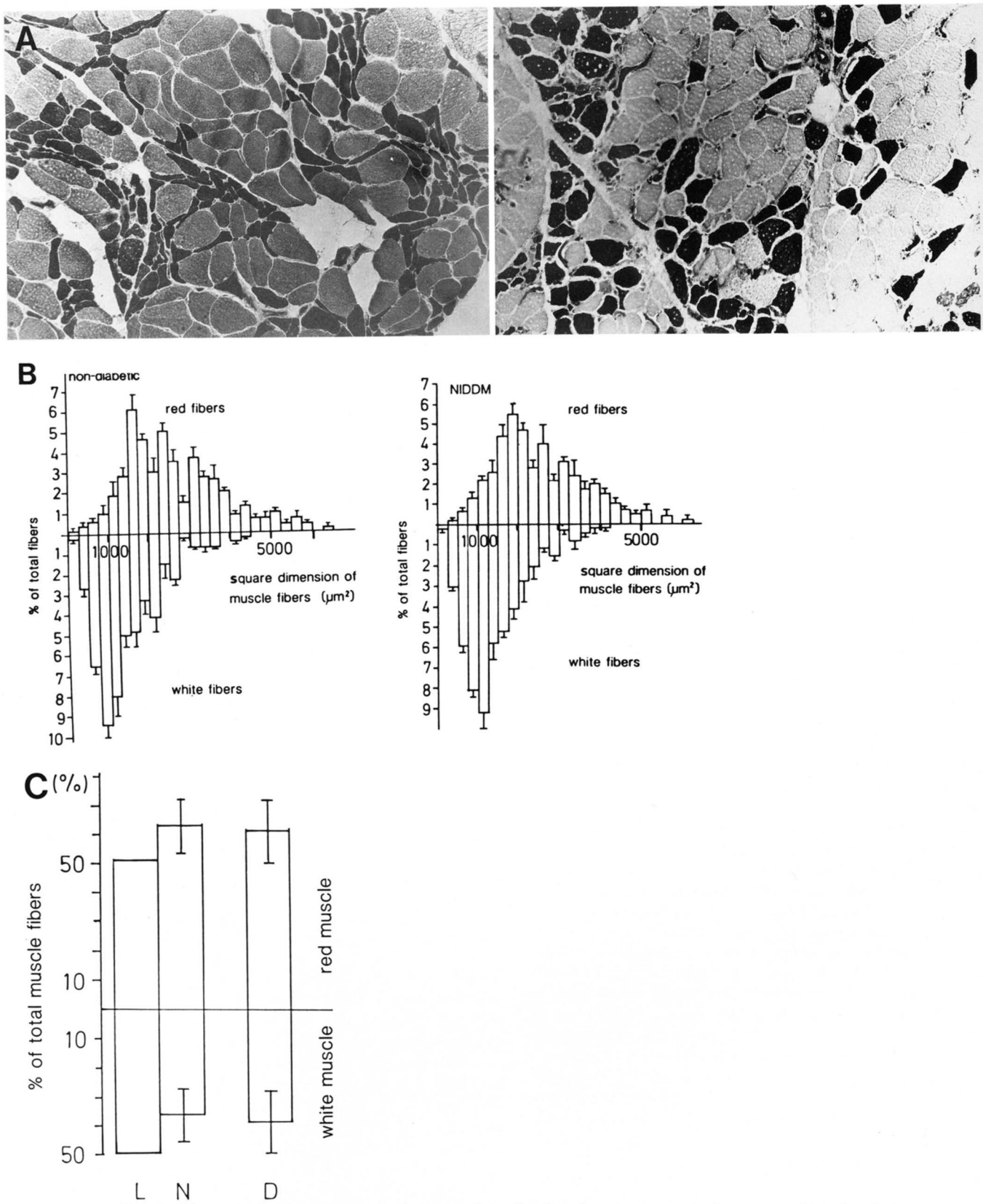

FIG. 1. **Characterization of muscle tissue probes.** *Panel A* shows a representative enzyme histochemical preparation from non-diabetic (*left*) and diabetic (*right*) m. gastrocnemius (× 100). The tissue was stained with myofibrillic ATPase (pH 9.4). The bright cells are type I red muscle fibers, and the dark cells are type II white muscle fibers. The pictures show neurogenic atrophy according to peripheral vascular neuropathy with grouped atrophy especially in type II fibers, type-grouping of the type I fibers. *Panel B* shows histograms of square sections of m. gastrocnemius from non-diabetic (*left*, $n = 8$) and diabetic patients (*right*, $n = 9$). Means ± S.D. of each square dimension class for red and white muscle fibers are shown. *Panel C* shows a comparison of total square section from the histogram depicted in *panel B* for non-diabetic (*N*) and diabetic (*D*) m. gastrocnemius with that derived from normal fiber composition of m. lattissimus dorsi (*L*) as published in Ref. 50.

TABLE II

Muscle enzyme activities and non-collagen protein (NCP) levels for patients admitted into the study

The enzyme activities were measured twice for each patient as described under "Experimental Procedures." The units of activity were normalized for non-collagen protein. Muscle probes which revealed activities which were not within the given ranges were excluded from further characterization of the receptor kinase.

Enzyme	Nondiabetic muscle	NIDDM muscle
	units/g NCP	*units/g NCP*
Lactate dehydrogenase	1040 ± 290	1070 ± 340
Phosphofructo kinase	150 ± 40	155 ± 30
Phosphoglycerate kinase	880 ± 200	910 ± 270
Phosphoglucomutase	975 ± 175	1060 ± 260
	mg/g muscle	*mg/g muscle*
NCP	76 ± 16	72 ± 10

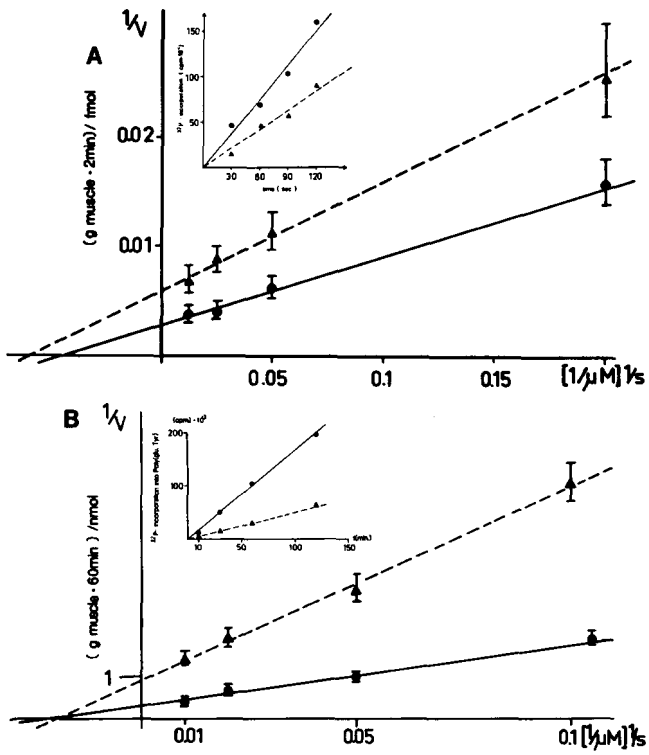

FIG. 6. **Kinetic data for autophosphorylation and substrate phosphorylation activity.** *Panel A*, a Lineweaver-Burk plot showing the relation between autophosphorylation and ATP concentration for control (●) and diabetic (▲) receptors. These data were obtained during a 2-min incubation, during which time autophosphorylation was linear (*inset*). *Panel B*, a Lineweaver-Burk plot showing the phosphorylation of poly(Glu:Tyr 4:1) by the control (●) and diabetic (▲) receptor as a function of poly(Glu:Tyr) concentration. These data were obtained as described in the method section for a time period of 60 min, during which the phosphorylation was linear (*inset*). Mean ± S.D. are presented for the data of all patients in group I (●) and group II (▲).

the regulatory region or the C terminus do not play a direct role in activation, as previously suggsted (51, 53).

DISCUSSION

The insulin receptor kinase isolated from the skeletal muscle of NIDDM patients exhibits *in vitro* a reduced insulin sensitivity and responsiveness (32–34). Our results agree in general with the findings in human fat (31) and liver cells (54) of type II diabetic patients and suggest that a kinase abnormality might contribute to the pathogenesis of insulin

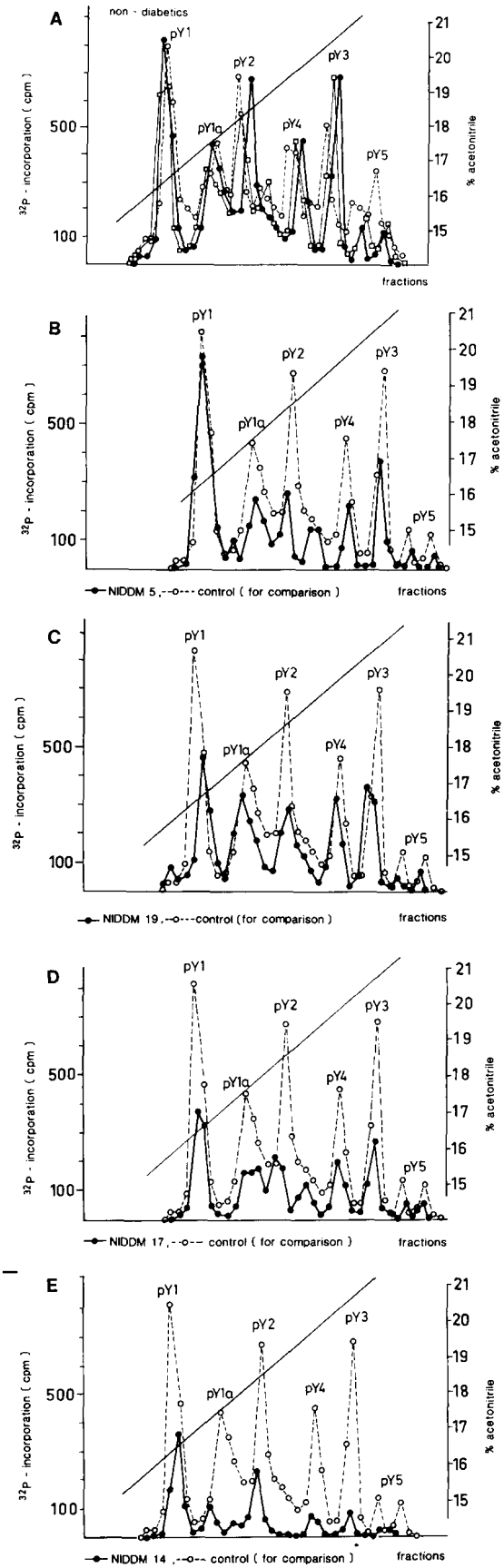

FIG. 7. **HPLC-profiles of tryptic peptides from several nondiabetic (*A*) and diabetic receptors (*B–E*).** Equal amounts of insulin receptor were phosphorylated for 10 min during insulin stimulation (controls, 100 nM and diabetic 1000 nM), and processed as described under "Experimental Procedures." About 94% of the radio-

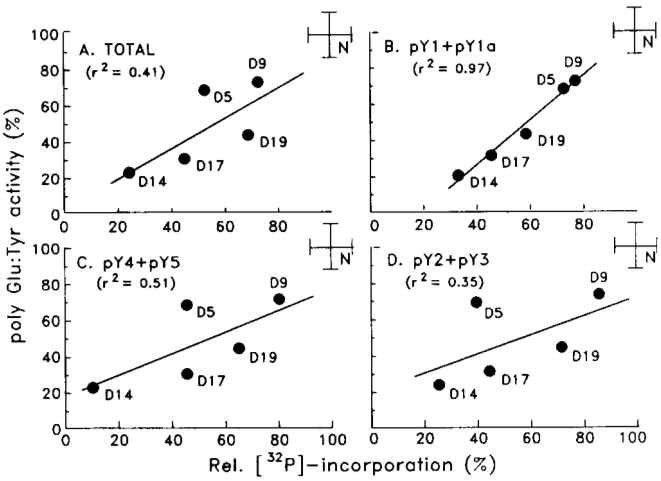

FIG. 8. **Association of poly(Glu:Tyr 4:1) activity and autophosphorylation of the β-subunit of six non-diabetic receptors (*N*) and five diabetic (*D5–D19*) receptors.** Poly(Glu:Tyr) activity of each patient receptor is represented as a percentage of the mean activity of the control receptors. Mean ± S.D. for poly(Glu:Tyr) activity and ^{32}P incorporation into the different HPLC peaks of six non-diabetic preparations are used as 100%. Autophosphorylation of each phosphopeptide from the diabetic receptor is represented as a percentage of the average phosphorylation observed with the controls. *Panel A* correlates the relative activity of each patient sample to the total autophosphorylation calculated as the sum of pY1, pY1a, pY2, pY3, pY4, and pY5. The other panels (*B–D*) correlate the relative activity of the diabetic receptors to tris-phosphorylation (*pY1 + pY1a*) and bis-phosphorylation of the regulatory region, and phosphorylation of the C terminus (*pY2 + pY3*), respectively. The substrate phosphorylation was measured and standardized as described in the legend of Fig. 5. The correlations were calculated by the least squares method.

resistance in type II diabetes. However, Caro *et al.* (37) did not find a diabetes-specific defect in human skeletal muscle, but the results were difficult to interpret because the kinase activity was studied in extremely obese patients. The extreme overweight of these patients might produce alterations of the receptor kinase which mask a diabetes-specific defect. Therefore, we provisionally conclude that a kinase abnormality exists in type II diabetes which contributes to the pathogenesis of the disease.

Since the muscle fiber composition was not altered during NIDDM, the reduced activity of the skeletal muscle insulin receptor is probably not due to alterations of the tissue composition. The receptor from NIDDM patients shows no change of insulin binding, suggesting that the reduced kinase activity is not caused by receptor down-regulation (55–58) or a structural alteration of the binding domain in the α-subunit of the insulin receptor. The diabetic receptor showed no change of affinity for ATP in both auto- and substrate phosphorylation. However, during insulin stimulation the V_{max} for autophosphorylation and poly(Glu:Tyr) phosphorylation was reduced. Furthermore, the stimulation of autophosphorylation by insulin was less sensitive for the diabetic receptor. Therefore, the reduced kinase activity observed in the muscle of NIDDM patients may be due to altered coupling between insulin binding and the particular autophosphorylation events which activate the kinase.

Separation of the tryptic phosphopeptides from the β-subunit of the diabetic receptor revealed an elution pattern qualitatively similar to the control. However, the level of phosphorylation of each peptide was variable in the NIDDM-derived receptors which may partially explain the variable kinase activity found during the poly(Glu:Tyr) assay. Several tyrosine residues in the β-subunit are involved in the autophosphorylation reaction including Tyr-1146, 1150, and 1151 in the regulatory region, and Tyr-1316 and Tyr-1322 in the C terminus (51, 64, 65). Autophosphorylation proceeds through a sequential mechanism in which Tyr-1146 and either Tyr-1150 or Tyr-1151 are phosphorylated first, generating a bis-phosphotyrosyl region which can be trapped by the addition of anti-phosphotyrosine antibodies (51, 66). However, this partial autophosphorylation does not activate the phosphotransferase, and complete autophosphorylation of all three tyrosyl residues in the regulatory region forming a tris-phosphotyrosyl domain is required for full activation (51).

The degree of activity of the receptor kinase toward poly(Glu:Tyr) does not correlate well with the overall level of ^{32}P incorporation into the β-subunit of the receptor. An even weaker association was found between activity and phosphorylation of the C terminus, which is consistent with previous results suggesting that phosphorylation of this region does not activate the kinase (53, 67). Moreover, bis-phosphorylation of the regulatory region is poorly associated with activation of the kinase. In contrast, tris-phosphorylation of the regulatory region is closely associated with kinase activation. These results support the conclusion that autophosphorylation of Tyr-1146, 1150, and 1151 is required to activate the kinase of insulin receptor (51). Furthermore, these data suggest that the defect of the insulin receptor kinase in NIDDM is located within the receptor itself, and alters the intramolecular autoactivation cascade which occurs during insulin stimulation.

Based on the present data, it is not possible to favor a particular molecular defect which inhibits the autophosphorylation cascade. Serine phosphorylation of the β-subunit decreases its tyrosine kinase activity, but it is unlikely to play a role in our NIDDM patients. Serine phosphorylation stimulated by phorbol esters causes an increase of the K_m for ATP in rat adipocytes (59–61), a change which we did not observe. Although serine phosphorylation causes a decrease in the V_{max} for autophosphorylation and kinase activity in Fao hepatoma cells (62), it blocks autophosphorylation proportionally at all of the sites, which is not the case for the receptors from NIDDM patients. Another possible cause for a kinase alteration is extreme hyperinsulinemia. It was recently demonstrated *in vitro* that high insulin concentrations (100 nM) reduce the kinase activity by changing both K_m and V_{max} (63). However, it is unlikely that hyperinsulinemia causes the type of alterations of the kinase observed here. On the one hand, the insulin concentrations found in our NIDDM patients are at least two orders of magnitude lower than the insulin concentrations used in this study (63), and on the other hand, we found no change in K_m.

In summary, we have characterized *in vitro* the kinase activity of the insulin receptor from skeletal muscle of several NIDDM patients and compared the activity with receptor from control patients. The muscle composition from all the patients was identical, but activation of the tyrosine kinase from the NIDDM patients was reduced. The relative activity of these defective receptors correlated strongly with the level of tris-phosphorylation of the regulatory region of the β-

activity was recovered from the HPLC-column. The absolute incorporated ^{32}P activity is depicted for the different patients, standardized on the same specific activity. *Panel A* shows profiles from two different non-diabetic m. gastrocnemius preparations (●, ○) and one m. latissimus dorsi preparation (□). The coefficient of variability for the single peaks was 10.4 ± 1.9%. *Panels B–E* show profiles from four different diabetic patients (●) compared with a representative non-diabetic (○).

subunit, that is Tyr(P)-1146, Tyr(P)-1150, and Tyr(P)-1151. Thus, we conclude that the autophosphorylation cascade of the β-subunit that activates the tyrosine kinase is inhibited in the patient receptors. We speculate that an amino acid exchange in the regulatory region of the β-subunit might prevent efficient transition from the bis-phosphorylated to the tris-phosphorylated state. Under sufficient physiological stress, this mutant receptor form may manifest itself as NIDDM. Further studies of this region of the β-subunit hopefully will allow us to further identify this regulatory defect.

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Supplementary Material to

A Defective Intramolecular Autoactivation Cascade May Cause the Reduced Kinase Activity of the Skeletal Muscle Insulin Receptor From Patients with NIDDM[1]

by

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EXPERIMENTAL PROCEDURES

Materials - Porcine [^{125}I-Tyr^{A14}] insulin (2200 Ci/mmol) and [γ-^{32}P]-ATP (3000 Ci/mmol), were from New England Nuclear (Dreieich, FRG); aprotinin, phenylmethylsulfonyl fluoride (PMSF), poly(Glu 4: Tyr 1), leupeptin, pepstatin, bensamidin, bacitracin and leucine were from Sigma (Munich, FRG). Wheat germ agglutinine coupled to agarose was from Miles (ICN, Eschwege, FRG); Triton X-100, all reagents for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and the protein assay were from Bio-Rad (Munich, FRG); and all other reagents were of the best grade commercially available. For autoradiography X-OMAT AR5 diagnostic film was used from Kodak (Rochester, New York). The tissue disintergrator Mikro-Dismembrator II was from B. Braun, Melsungen, FRG. HPLC-equipment together with C18 reversed-phase column TSK ODS-120T 5 lm was from LKB (Freiburg, FRG), and the RP-318 was reversed phase column from Bio-Rad (Munich, FRG).

Selection of the tissue probes - Three groups of patients were chosen: One diabetic and one non-diabetic group of 60 to 80 years old patients who had to undergo an amputation of the leg as a consequence of peripheral arteriovascular complications and another non-diabetic group of 40 to 50 years old patients who had to undergo a muscle transplantation for plastic reconstructive reasons. Tissue specimens of musculus gastrocnemius were taken from the patients of the first two groups, and specimens of musculus latissimus dorsi were taken from the third group. Informed consent was obtained from all patients. All the probes were taken right away from the operating table, cut into small pieces, immediately frozen in liquid nitrogen and stored at -70°C.

The muscle samples were used for further studies after a selection procedure which included the following criteria: 1. light microscopic, electron microscopic and histochemical qualities of the tissue, 2. enzymatic activity of lactate dehydrogenase EC 1.1.1.27 (LDH), phosphofructokinase EC 2.7.1.11 (PFK), phosphoglycerate kinase EC 2.7.2.3 (PGK) and phosphoglucomutase EC 2.7.5.1 (PGM) and 3. non-collagen protein (NCP) in the normal range for the muscle tissue.

Histological, histochemical and morphometric examination - Two aliquots of every tissue specimen were fixed in formaldehyde or Heidenhain-solution (38). The third was placed on a cork piece by freezing in liquid nitrogen. Out of these probes the following staining and histochemical reactions were performed: haematoxilin eosin staining (38) periodic acid Schiff-reaction (38), oilred-o-staining (39), myofibrillary ATPase pH 9.4 (40), NADH-tetrazolium-reductase (41) and myo-phosphorylase (42). In probes with still unresolved histological questions electron microscopy was done. To evaluate the fiber composition as Type I/Type II-ratio the ATPase stained probe was examined by taking a photograph and evaluation of the projected image using a morphometric equipment from Leitz, West Germany.

Muscle enzyme activities - Frozen tissue specimen of 30 mg were homogenized by a tissue disintegrator and suspended in 600 11 of 50 mM Hepes buffer containing 1 mM EDTA, pH 7.5 at 4°C; aliquots of this suspension were clarified by a 15 min centrifugation at 8000 xg and washed once more with the same volume of extraction buffer. Both supernatants were put together and used for the enzyme and NCP assays. The assays were carried out as described (43,44). Enzyme activities are depicted as international units: 1 U = 1 lmol/min.

Partial purification of insulin receptor - The skeletal muscle samples (8-15 g of tissue) were homogenized by an ultra-turrax for 10-15 s with a maximal speed at 4°C in the presence of the protease inhibitors PMSF (5 mM), aprotinin (1200 trypsin inhibiting units (1), leupeptin (2 lM), pepstatin (2 lM), benzamidin (10 mM), bacitracin (7500 U/l), leucine (10 mM) and EDTA (5 mM) in a buffer containing NaH_2PO_3 (10 mM). All further steps were performed as described in (45).

Binding to solubilized receptor - Solubilized and wheat germ purified receptor was prepared as described above. Aliquots of the wheat germ eluate containing 5 lg of protein were incubated with [^{125}I-Tyr^{A14}]-insulin (3 x 10^{-11} M, 3000 cpm/fmol) and various concentrations of unlabeled insulin for 45 min at 22°C in a medium of 25 mM tris (pH 7.4), 5 mM $MgSO_4$ and 0.5 g% bovine serum albumin (BSA). Separation of the free and receptor-bound insulin was then performed using dextrane-coated charcoal. Scatchard analysis was done with a computer program by G.A. McPherson 1985 (46).

Autophosphorylation of the insulin receptor - For the standard phosphorylation assay approximately 10 lg of wheat germ purified proteins were preincubated at 22°C for 30 min with insulin (10^{-10} M - 10^{-6} M) or without insulin. This was followed by an incubation with [c-^{32}P]-ATP (5 lM, 0.01 - 0.1 mCi) if not otherwise stated, in 25 mM Hepes buffer (pH 7.4), containing 0.1% (by vol.) Triton X-100, 10 mM $MnCl_2$, 12 mM NaCl, 0.5 mM KCl, 0.25 mM $CaCl_2$, 0.1 mM KH_2PO_4, 0.1 mM $MgSO_4$, 0.25 g% BSA, and 1 mM vanadate at 22°C for the times indicated in the figure legends. The incubation was stopped by addition of Laemmli buffer containing 100 mM dithiothreitol and 80 mM $NaDodSO_4$ and boiling for 30 min. Subsequently phosphoproteins were separated by polyacrylamide gel electrophoresis (47). The phosphoproteins identified by the autoradiography were cut from the gel and counted in a scintillation counter.

Substrate phosphorylation - Substrate phosphorylation activity was analyzed by a modification of the method of Braun S. et al. (48). Approximately 2 lg of wheat germ purified proteins were preincubated with insulin (10^{-9} M and 10^{-7} M) and without insulin for 30 min at 22°C. This was followed by an incubation with [c-^{32}P]-ATP (50 lM, 0.005 mCi) in the final volume of 50 11 containing 25 mM Hepes buffer, containing 0.1% (by vol.) Triton X-100, 12 mM MgCl, 12 mM MnCl, 1 mM vanadate, and 0.5 mM Poly(Glu 4:Tyr 1) for 1 hour at 22°C. (The kinetic studies for this substrate phosphorylation assay revealed a straight line crossing through the axial section). The phosphorylation was stopped by application of the reaction mixture to Whatman 3MM paper squares washed with 20 mM sodium pyrophosphate. The papers were washed 6 times in 10% (by vol.) TCA containing 20 mM sodium pyrophosphate and counted with a scintillation mixture in a b-counter.

Tryptic peptide mapping - Tryptic peptide mapping by high performance liquid chromatography was performed as described earlier (49). Fixed, stained, destained, and dried polyacrylamide gel fragments containing the phosphorylated 95 kDa b-subunit of the receptor located by autoradiography were washed for 12 h at 37°C with 20 ml of 10% methanol. The gel was dried at 70°C for 3 h and rehydrated in 600 11 of 50 mM NH_4HCO_3 containing 30 lg of L-1-tosylamide-2-phenylethyl chloromethyl ketone treated trypsin. The mixture was incubated for 2 x 12 h at 37°C, the gel fragment was removed, and the supernatant was clarified by centrifugation. The supernatant was lyophilized and the residue was dissolved in 25 - 50 11 of 0.1% trifluoroacetic acid and once more clarified by centrifugation and filtration. The phosphopeptides were separated using an high performance liquid chromatography system equipped with a LKB TSK-ODS or Bio-Rad RP-318 reversed-phase column. Phosphopeptides applied to the column were eluted with water containing 0.05% trifluoroacetic acid and modified with acetonitrile, also containing 0.05% trifluoroacetic acid (LKB-column: flow rate of 0.8 ml/min, 0% acetonitrile for 5 min, 0 - 40% acetonitrile in 40 min, 5 drops per fraction collected; Bio-Rad column: flow rate 1.1 ml/min; 5% acetonitrile for 5 min, 5 25% acetonitrile in 80 min, 1 fraction per minute). Cerenkov radiation of 0.1 ml to 0.16 ml respectively of 1.1 ml fractions eluted from the columns was measured.

RESULTS

Insulin binding to the WGA purified insulin receptor from normal and NIDDM tissue--- The binding properties of the WGA-purified insulin receptors isolated from muscle of non-diabetic (Group I) and NIDDM patients (Group II) were determined. No significant differences were observed in the displacement curves or the Scatchard plots of each group (Fig. 2). Thus the affinity and binding capacity of the insulin receptor isolated from the skeletal muscle of the NIDDM patients were within the normal range.

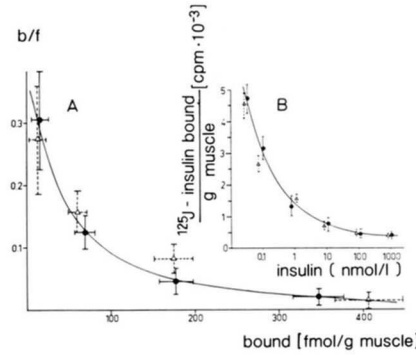

Figure 2. Insulin binding characteristics of solubilized insulin receptor from NIDDM muscle (△) and control muscle (●) obtained as described under experimental procedures. The binding data for all of the samples (Table I) are shown as displacement curves (insert) and Scatchard plots (Means±SD).

Insulin receptor autophosphorylation and kinase activity---The insulin receptor purified from skeletal muscle on WGA-agarose from control or NIDDM patients was incubated with [γ-^{32}P]ATP in the absence or presence of insulin. Equal amounts of receptor were used in each assay as determined by the insulin binding capacity of each preparation. After phosphorylation, the insulin receptor was immunoprecipitated with an anti-insulin receptor antibody and separated by SDS-PAGE. A representative autoradiogram of the phosphorylated β-subunit (M_r=95 kDa) is shown for the non-diabetic (Fig. 3, top-left) and NIDDM muscle (Fig. 3, top-right). The dose response curve for insulin-stimulated autophosphorylation of the β-subunit from NIDDM patients is shifted to the right relative to the control (Fig. 3, bottom). To detect this shift, the average data from each muscle sample were expressed as a percentage of maximal ^{32}P-incorporation into the β-subunit. Even though the diabetic group showed great individual variance, there is a significant difference between the two groups at 10^{-8} M insulin.

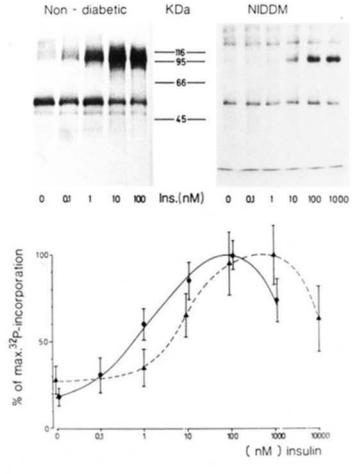

Figure 3. Autophosphorylation activity of non-diabetic and diabetic insulin receptor. Insulin receptor was phosphorylated as described under experimental procedures for 10 min, stopped and then applied to SDS-PAGE gels. Top panel: two representative autoradiograms (not exposed in parallel) showing the dose-response of insulin-stimulated ^{32}P-incorporation into the 95 kDa β-subunit of the insulin receptor from non-diabetic (*left*) and NIDDM muscle (*right*). Using the 50 kDa band as an insulin independent internal standard a 2.5 times higher ^{32}P-incorporation was found on an average in the maximal stimulated 95 kDa band for the non-diabetic group and a 1.7 times higher for the diabetic. The coefficient of variability for the autophosphorylation assay using the same receptor was 8.5%. *Bottom panel*: The average dose-response curves for the non-diabetic (●) and diabetic (▲) groups (See Table I) as % of maximal [^{32}P]phosphate incorporation (means±SD).

The total phosphate incorporated into the receptor β-subunit for each patient is summarized in Figure 4. There was no difference in basal autophosphorylation of the receptor from non-diabetic and NIDDM patients. In contrast, during maximal insulin stimulation a significant reduction in the extent of autophosphorylation was observed for the diabetic receptors. Autophosphorylation of the individual samples only reached 45 to 75% of the mean value of non-diabetic controls (group I and III combined), suggesting that the reduction of the insulin effect occurred in all of the samples, but the magnitude was variable.

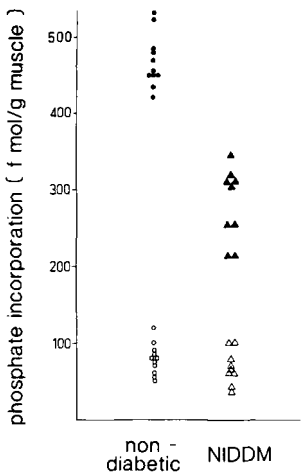

Figure 4. Phosphate incorporation into the 95 kDa β-subunit of the insulin receptor of each patient from group I and II, and of each preparation for group III. Autophosphorylation was carried out as described in Fig. 3 and under experimental procedures and total phosphate incorporation was calculated for the basal and maximal stimulated probes (10^{-7} or 10^{-6} M insulin). (controls: basal (○) and maximal stimulation (●); NIDDM: basal (△) and maximal stimulation (▲)).

In order to detect possible influences of age, localization of the muscle, influences of the surgical technique or undetected arteriovascular disease, we studied the insulin receptor kinase of musculus latissimus dorsi of the group III patients. The maximal [^{32}P]phosphorylation was within the range of the non-diabetic control group I. These values are included in Fig. 4 with the group I controls. Furthermore, the dose response and the maximal ^{32}P-incorporation of the kinase isolated from the gastrocnemius of the 8 patients of group 1 was identical with the dose response found for the kinase isolated from the musculus lattissimus dorsi of the younger patients of group III (data not shown). These values also agreed with previously published results (45). Therefore, in addition to the morphological and enzymatic examination of the tissue samples (Fig. 1, Table 2), the results with group III further exclude nonspecific effects due to the surgical technique, the age of the patients, the muscle localization and the immobilization of hospitalized patients.

Kinetic properties of the insulin receptor kinase---The kinase activity of the insulin receptor purified from muscle of NIDDM patients was significantly less than the activity of the control during assays with poly(Glu:Tyr 4:1). Similar to the autophosphorylation activity, poly(Glu:Tyr) phosphorylation during maximal insulin stimulation was reduced about 50 - 80% (Fig. 5). Although basal autophosphorylation was similar for control and NIDDM receptors, a significant difference was detected for basal phosphorylation of poly(Glu:Tyr).

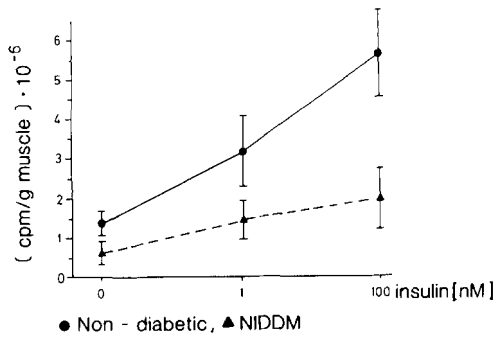

Figure 5. Insulin dose-response curve of poly(Glu:Tyr 4:1) phosphorylation for group I non-diabetic (●) and group II diabetic receptors (▲). The substrate phosphorylation assay was carried out with each muscle sample as described under experimental procedures for 1 h using 1.8 mM poly(Glu:Tyr 4:1). The kinase activity was measured in the absence of insulin, and at 1 and 100 nM insulin using equal amounts of insulin receptor as determined by insulin binding. The average activity (±SD) for each group (Table I) is shown. The coefficient of variability for the assay using the same receptor was 5%.