Insulin-Receptor Kinase Activity of Adipose Tissue From Morbidly Obese Humans With and Without NIDDM

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SUMMARY

We have determined glucose transport, insulin binding, and insulin-receptor kinase activity in adipose tissue from morbidly obese patients with and without non-insulin-dependent diabetes mellitus (NIDDM). The insulin sensitivity and responsiveness of glucose transport in freshly isolated adipocytes were significantly reduced in NIDDM subjects compared with nondiabetics. This was due in part to decreased insulin binding in adipocytes. Reduced specific ^{125}I-labeled insulin binding was also observed in crude detergent extracts and partially purified insulin receptors from adipose tissue. In addition, the basal and insulin-stimulated tyrosine-specific protein kinase activity per milligram of protein was significantly decreased in NIDDM patients compared with nondiabetics. The differences between maximally insulin-stimulated and basal kinase activities expressed by insulin-binding activity were also significantly reduced in NIDDM subjects. We conclude that insulin resistance in morbidly obese patients with NIDDM is due to both insulin-binding and postbinding defects. One of the postbinding defects in NIDDM appears to be impaired insulin-receptor kinase activity of fat tissue. *Diabetes* 36:620–25, 1987

Insulin resistance in patients with non-insulin-dependent diabetes mellitus (NIDDM) has been ascribed to both insulin-binding and postbinding defects (1). Kasuga et al. (2) and others (3) have shown that insulin promotes autophosphorylation of the β-subunit of the insulin receptor and also stimulates the phosphorylation of various exogenous substrates. Although a specific physiological intracellular substrate(s) for insulin-receptor kinase has not been clearly defined (4,5), several laboratories, including our own, have studied insulin-receptor kinase activity in various insulin-resistant animals models (6,7) and in patients with extreme insulin resistance (8) to define the possible loci of the defect(s) in insulin action. In our study, we demonstrate a significant defect in tyrosine-specific protein kinase activity of insulin receptors in adipose tissue from morbidly obese NIDDM subjects when compared with nondiabetic morbidly obese subjects. However, our results do not imply an irreversible or intrinsic change in receptor function in NIDDM, because the changes may well be due to metabolic abnormalities related to diabetes.

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MATERIALS AND METHODS

Patients. Clinical and biochemical data from 17 nondiabetic morbidly obese subjects and 14 morbidly obese subjects with NIDDM are shown in Table 1. The classification of subjects with normal glucose tolerance and the diagnosis of diabetes was made by the National Diabetes Data Group criteria. The patients were admitted 4 days before gastric bypass surgery. During this period, they remained active at approximately prehospitalization exercise level and received a weight-maintaining diet (50% carbohydrate, 30% fat, and 20% protein). Insulin or oral hypoglycemic agents were discontinued before admission. Rapid-acting insulin was used only if the preprandial plasma glucose was >300 mg/dl. The subjects underwent surgery after overnight fast. Only intravenous saline was given before removing abdominal (above umbilicus) subcutaneous fat (10–15 g) that was obtained from the surgical incision immediately after exploring the abdominal cavity. Informed written consent was obtained from all patients after explanation of nature and potential risks of the study.

Isolation of adipocytes. Fat cells were isolated by the method of Rodbell as modified by Pedersen et al. (9) for isolating human adipocytes. Subcutaneous fat tissue was cut into small pieces (1–2 mm) and digested with collagenase (0.5 mg/ml; type I CLS, Worthington, Freehold, NJ) in

TABLE 1
Clinical and biochemical data of patients studied

	Nondiabetic	NIDDM
Number	17	14
Sex		
Female	15	12
Male	2	2
Age (yr)	36 ± 3	40 ± 2
Weight (kg)	122 ± 5	127 ± 5
Height (cm)	167 ± 3	166 ± 2
Body fat (%)	48 ± 1	49 ± 1
Adipocyte diameter (μm)*	105 ± 5	106 ± 4
Adipocyte volume (pl)*	772 ± 62	803 ± 94
Fasting glucose (mg/dl)	87 ± 3	212 ± 18†
Rate of glucose disappearance (%/min)‡	1.2 ± 0.1	0.5 ± 1†
Fasting insulin (μU/ml)	35 ± 5	39 ± 5
Duration of obesity (yr)	17 ± 4	20 ± 4
Duration of diabetes (yr)		3.2 ± 0.8
Treatment of diabetes (no. of patients)		Insulin (3) Sulfonylureas (5) None (6)

*Mean values from 5 subjects.
†$P < .01$.
‡After intravenous glucose tolerance test (25 g).

Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4., containing 5 mM glucose for 45–60 min at 37°C under 95% O_2/5% CO_2 atmosphere at 150 rpm in a circulating bath. Cells were filtered through nylon mesh and washed with 3% bovine serum albumin (BSA)–KRB buffer. The cells were suspended at 20% lipocrit in 3% BSA-KRB buffer for determining ^{125}I-labeled insulin binding and glucose transport. A 5-μl aliquot of cell suspension (10% lipocrit) in 10% formalin-buffered saline, fixed overnight, was counted for cell number and size under the microscope with a grid in the eyepiece. Lysis of adipocytes was negligible, and no preferential breakage of larger fat cells was observed during the isolation procedure.

Insulin binding in adipocytes. Freshly isolated adipocytes at 20% lipocrit (~200,000 cells/ml) were incubated with ^{125}I-insulin (0.6 ng/ml) in the absence and presence of increasing concentrations of unlabeled insulin for 2 h at 22°C in 1 ml of 10 mM HEPES-KRB buffer, pH 7.4, containing 3% BSA and 2 mM glucose. Three 250-μl aliquots of incubation mixture were centrifuged through silicone oil, and the radioactivity associated with the cells was counted. The nonspecific binding was consistently <10% of the total binding, and the degradation of ^{125}I-insulin determined by TCA precipitation was also <10% under assay conditions. Because actual cell number was not immediately available, the insulin-binding experiments were performed with fixed lipocrit, and percent specific ^{125}I-insulin binding was normalized for 50,000 cells/0.25 ml.

Glucose transport in adipocytes. Glucose transport in fat cells was measured by determining the uptake of U-[^{14}C]glucose at tracer concentrations as described by Kashiwagi et al. (10). These investigators have validated this method for measuring glucose transport in comparison with 3-*O*-methylglucose uptake in nondiabetic and diabetic subjects (11). Basal and insulin-stimulated (10^{-10} and 10^{-7} M, respectively) glucose transport, as measured by two different methods that use U-[^{14}C]glucose (10) and ^{14}C-labeled 3-*O*-methylglucose (12) isotopes, were comparable in our morbidly obese subjects with or without NIDDM.

The fat cells (~200,000 cells/ml) were incubated with 500 nM U-[^{14}C]glucose (260 mCi/mmol; New England Nuclear, Boston, MA) in the absence and presence of insulin (1×10^{-11} to 1×10^{-7} M) for 1 h at 37°C under 95% O_2/5% CO_2 atmosphere in 10 mM HEPES-KRB buffer, pH 7.4, containing 3% BSA. The cells were then separated by centrifuging through oil and ^{14}C radioactivity in cells counted.

Wheat germ agglutinin (WGA)–Sepharose–purified solubilized receptors. Adipose membranes were prepared as described by Livingston et al. (13) with minor modifications. The fat tissue (10 g) was cut into small pieces and then homogenized with 60 ml of 0.25 M sucrose-Tris buffer, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 2 μM pepstatin, and 2 μM leupeptin. After removal of nuclei, tissue debris, and fat plug, the 30,000 × *g* pellet representing total membranes was washed and then solubilized for 1 h at room temperature (22°C) in 1.0 ml of 1% Triton X-100 (Rohm & Haas, Philadelphia, PA)–50 mM Tris buffer, pH 7.4, containing 1000 U/ml aprotinin, 1 mM vanadate, 0.1 mM PMSF, 0.1 mg/ml bacitracin, 2 μM leupeptin, and 2 μM pepstatin. The insoluble material was removed by centrifugation at 100,000 × *g*. This

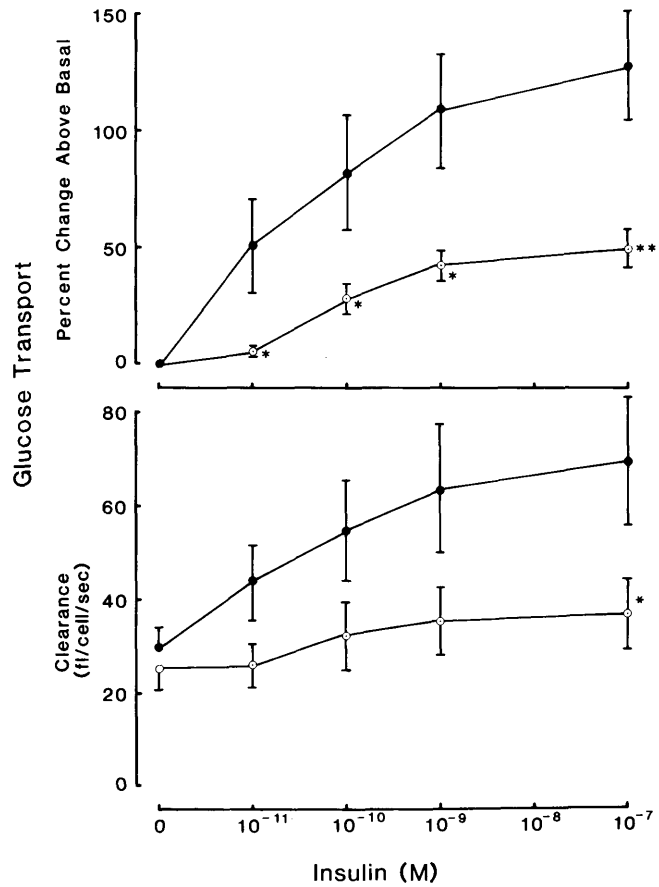

FIG. 1. Glucose transport at tracer U-[^{14}C]glucose concentration (500 nM) in absence and presence of insulin in freshly isolated adipocytes from morbidly obese nondiabetic (●; *n* = 8) and NIDDM (○; *n* = 6) subjects. *Lower panel* represents glucose transport values in absence and presence of different insulin concentrations. *Upper panel* represents percent change above basal glucose transport. Each *point* represents mean ± SE. *$P < .05$, **$P < .01$.

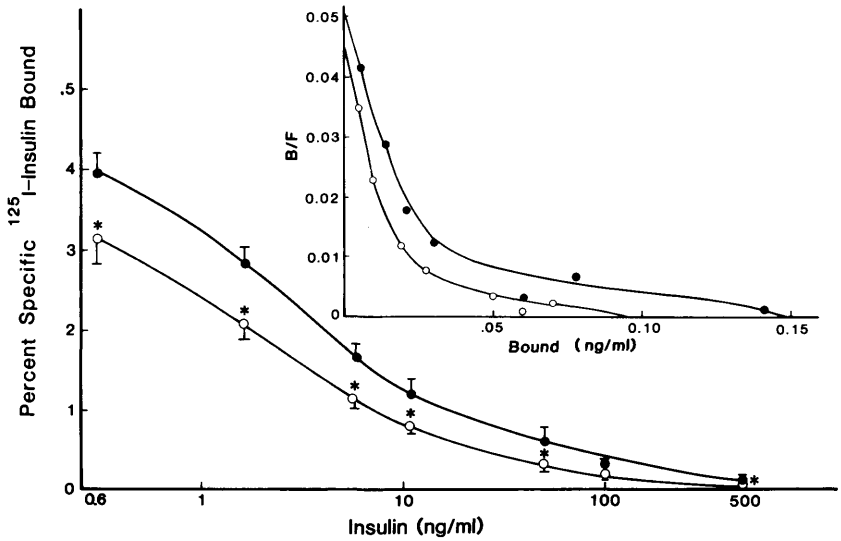

FIG. 2. The binding curves of specific ^{125}I-insulin binding in freshly isolated adipocytes (50,000 cells/0.25 ml) from morbidly obese nondiabetic (●; *n* = 5) and NIDDM (○; *n* = 5) subjects. Scatchard plots are shown in *inset*. Each *point* represents mean ± SE. *$P < .05$.

crude detergent-solubilized extract was purified on a WGA-Sepharose column as previously described (14). The extensive washing of the insulin receptor bound to WGA-Sepharose column with 50 times its bed volume and the inclusion of protease and phosphatase inhibitors are important to minimize the contaminating activities of phosphoprotein phosphatases, proteases, and ATPases that might otherwise complicate interpretation of data.

Insulin binding. The method for determining ^{125}I-insulin binding in crude or WGA-Sepharose–purified detergent-solubilized extracts was previously described (14).

Tyrosine-specific protein kinase activity of insulin receptors. The tyrosine-specific protein kinase activity of insulin receptors was determined by the modified method of Grunberger et al. (8). In brief, 75-μl aliquots of WGA-Sepharose–purified solubilized receptors (~5 μg protein) were incubated for 16 h at 4°C in the absence and presence of insulin (1×10^{-10} to 1×10^{-7} M). Then, γ-[^{32}P]ATP (100 μM) was added in the presence of 2.5 mg/ml exogenous substrate Glu^{80}-Tyr^{20}, 10 mM $MgCl_2$, and 0.5 mM $MnCl_2$. The final reaction volume was 200 μl. After 30 min at room temperature (22°C), the reaction was stopped by adding 2.5 ml of 10% TCA containing 10 mM pyrophosphate, and then 100 μl of 1% BSA was added. The precipitated proteins were washed 4 times with 10% TCA and the final pellet dissolved in 1 ml of 0.5 N NaOH and counted in liquid scintillation counter. The incorporation of [^{32}P]ATP into exogenous substrate was linear in partially purified receptor preparations from both groups up to 60 min. The kinase activity also increased in a dose-dependent manner with increasing protein concentrations (1.25–10.0 μg protein) of partially purified receptors. The incorporation of radiolabeled phosphate was similar among 0.5-, 1-, 2.5-, and 5.0-mg/ml concentrations of exogenous substrate Glu^{80}-Tyr^{20} in the assay.

Statistical analysis. Student's *t* test, paired and unpaired, was performed to determine the level of significance.

RESULTS

The basal glucose transport expressed as glucose clearance rate in freshly isolated adipocytes was similar in both nondiabetic (29.1 ± 4.8 fl · cell^{-1} · s^{-1}, mean ± SE) and NIDDM (24.9 ± 5.4) subjects. Insulin significantly stimulated basal glucose transport at 1×10^{-11} M concentration in control and at 1×10^{-10} M concentration in diabetic subjects. At maximal insulin concentration, the glucose transport was significantly decreased in NIDDM (36.4 ± 8.2) compared with that in control (69.5 ± 14.3) subjects. The percent change above basal glucose transport at every insulin concentration used was significantly higher ($P < .05$ and $P < .01$) in the nondiabetic group compared with the NIDDM group (Fig. 1). The ED_{50} of insulin for glucose transport in obese controls ($0.41 \pm 0.24 \times 10^{-10}$ M) was significantly different ($P < .05$) from that in obese NIDDM subjects ($2.52 \pm 1.17 \times 10^{-10}$ M). For statistical analysis, we excluded the data from one patient with normal glucose tolerance because the ED_{50} of 1×10^{-9} M was higher than that from other control subjects (range $0.04–1.7 \times 10^{-10}$ M) and from even the diabetic subjects (range $0.25–8.0 \times 10^{-10}$ M). These results demonstrate decreased insulin sensitivity and responsiveness in NIDDM patients compared with age-, sex-, and weight-matched nondiabetic morbidly obese patients.

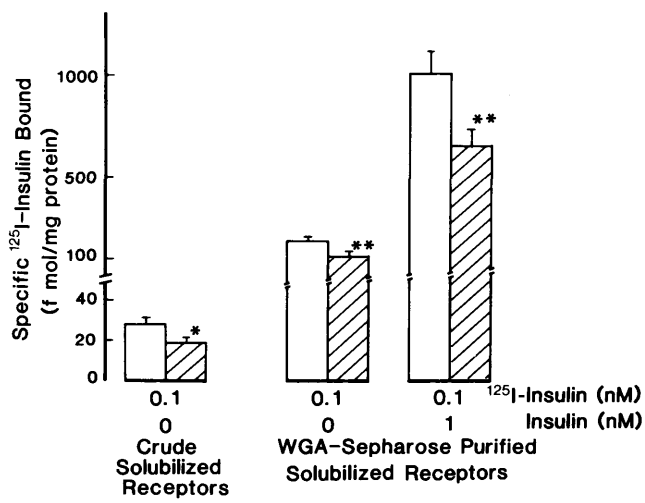

FIG. 3. Specific ^{125}I-insulin binding in crude or wheat germ agglutinin (WGA)–Sepharose–purified detergent-solubilized extracts of subcutaneous fat after incubation with tracer ^{125}I-insulin (0.1 nM) in absence or presence of insulin (1 nM) for 16 h at 4°C. Each *bar* represents mean ± SE from morbidly obese NIDDM (*hatched bars*; *n* = 9) and nondiabetic (*open bars*; *n* = 9) subjects. *$P < .05$; **$P < .01$.

TABLE 2
Insulin binding and insulin-receptor kinase activity in WGA-Sepharose–purified solubilized extracts of adipose tissue

Patient no.	^{125}I-insulin binding (fmol/mg protein)	Tyrosine-specific protein kinase activity (pmol/mg Glu^{80}-Tyr^{20} · min^{-1} · mg^{-1} protein) Insulin (M) 0	1×10^{-10}	1×10^{-9}	1×10^{-7}
Obese control					
1	182	5.04	5.36	5.86	8.68
2	152	4.54	5.39	5.21	6.29
3	114	6.62	7.25	7.74	8.83
4	242	19.10	23.50	28.80	39.50
5	180	22.30	25.00	31.00	36.20
6	136	22.70	25.30	26.50	27.00
7	152	11.47	13.26	17.67	17.20
8	175	14.57	15.65	16.20	17.02
9	206	7.89	10.11	12.98	16.45
Obese NIDDM					
1	54	4.93	5.17	5.68	5.36
2	108	5.78	5.86	6.07	7.62
3	73	6.72	5.76	7.18	6.66
4	118	6.01	6.47	6.58	7.22
5	143	7.02	7.50	7.60	7.60
6	108	9.95	9.80	10.60	12.43

The percent specific ^{125}I-insulin binding to fat cells was significantly higher ($P < .05$) in nondiabetics compared with diabetic subjects at various insulin concentrations (Fig. 2). The decrease in specific ^{125}I-insulin binding in diabetic subjects was mainly due to decreased receptor number. These differences were not due to differences in cell size in the two groups (Table 1) or in insulin degradation, which was <10% in both groups. The decrease in insulin receptors in fat cells from diabetics was also evident in the total pool of cellular receptors because the specific ^{125}I-insulin binding in crude solubilized receptors at tracer insulin concentration (1×10^{-10} M) was significantly ($P < .05$) decreased in NIDDM (18.7 ± 3.1 fmol/mg protein) in comparison with controls (28.4 ± 3.1) (Fig. 3). WGA-Sepharose chromatography of these crude solubilized receptors resulted in a six- to sevenfold increase in insulin-binding activity in both NIDDM and nondiabetic morbidly obese subjects. Similar to the observations in crude solubilized receptors, ^{125}I-insulin binding in WGA-Sepharose–purified receptors was significantly higher ($P < .01$) in controls than in diabetics (Fig. 3).

The decrease in insulin binding could explain reduced insulin sensitivity of glucose transport in NIDDM fat cells but not the reduced responsiveness of insulin-stimulated glucose transport. Therefore, we determined the tyrosine-specific protein kinase activity of insulin receptors from fat tissue. In Table 2, we have presented specific ^{125}I-insulin binding at tracer insulin concentration (1×10^{-10} M) and basal and insulin-stimulated tyrosine-specific protein kinase activity of partially purified insulin receptors from control and NIDDM groups. The basal kinase activity was significantly decreased in NIDDM (6.7 ± 0.8 pmol ^{32}P incorporated per mg protein · min^{-1} · mg^{-1} substrate) in comparison with nondiabetic subjects (12.7 ± 2.4; Fig. 4). Insulin significantly stimulated the basal tyrosine-specific protein kinase activity at 1×10^{-10} M concentration in the control group and at 1×10^{-9} M in the NIDDM group. The percent change above basal activity was significantly reduced in NIDDM in comparison with nondiabetic controls (Fig. 4). When the protein kinase activity was normalized for ^{125}I-insulin binding at tracer concentration, no significant difference was observed in basal activity in the two groups. In the absence of complete inhibition curves of specific ^{125}I-insulin binding in these re-

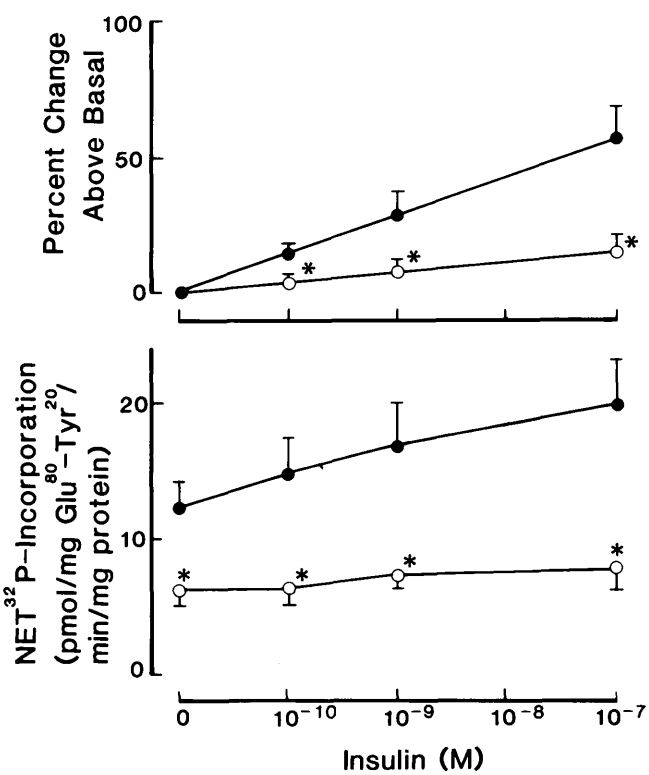

FIG. 4. Tyrosine-specific protein kinase activity of wheat germ agglutinin–Sepharose–purified detergent-solubilized extracts of subcutaneous fat in absence and presence of insulin. Each *point* represents mean ± SE from 6 morbidly obese NIDDM (○) and 9 nondiabetic (●) subjects. *$P < .05$.

ceptor preparations, insulin-receptor kinase activity at different receptor occupancies cannot be precisely determined. Considering these limitations, when we normalize insulin-stimulated kinase activity by specific ^{125}I-insulin binding at tracer concentration, no significant differences were observed between the two groups. However, the difference in the net activity at 10^{-7} M insulin concentration from basal activity per 100 fmol insulin-binding activity was significantly reduced in diabetics (1.04 ± 0.35) in comparison with nondiabetics (3.76 ± 0.89).

DISCUSSION

Our results, demonstrating decreased insulin sensitivity and responsiveness of glucose transport in fat cells from NIDDM patients, agree with previous studies (1,10,15). The decreased insulin sensitivity in diabetics appears to be partially due to reduced insulin binding (16,17). Although obesity per se is known to cause insulin resistance due to both binding and postbinding defects, this study further confirms that in type II diabetes, insulin sensitivity and responsiveness of glucose transport is even more deteriorated (15,18,19).

The postbinding mechanism of insulin resistance in type II diabetes is unknown mainly due to our poor understanding of postbinding events of insulin action. Recently, it was shown that insulin induces phosphorylation of the β-subunit of the insulin receptor and that the insulin receptor itself is a tyrosine-specific protein kinase with the ability to phosphorylate exogenous substrates and, presumably, also endogenous substrates (2–5). Thus, it has been hypothesized that insulin-receptor kinase might mediate insulin action. The phosphorylation of glucose transporters in rat fat cells has been shown to be modulated by insulin (20). Although no direct relationship between insulin-receptor kinase and phosphorylation of glucose transporters has been demonstrated, studying insulin-receptor kinase activity in NIDDM should shed light on the factors responsible for insulin resistance in type II diabetes.

Our results clearly demonstrate a defect in the ability of insulin to stimulate the tyrosine-specific protein kinase of insulin receptors in subcutaneous fat of morbidly obese NIDDM patients when compared with nondiabetic morbidly obese subjects. The diabetic patients are morbidly obese and relatively young. Therefore, our findings may not be representative of the entire NIDDM patient population. However, these findings are in close agreement with the impaired insulin-receptor kinase activity in livers (21) and skeletal muscles (22) of streptozocin-induced diabetic rats and the defective autophosphorylation of insulin receptors in skeletal muscle of hyperinsulinemic diabetic mice (23). In contrast, Amatruda and Roncone (24) observed normal insulin-receptor kinase activity in the livers from streptozocin-induced diabetic rats. Our study does not delineate the effect of obesity per se on the insulin-receptor kinase activity in adipose tissue because of the lack of a nonobese control group. However, we previously observed in human livers no significant alterations in insulin-receptor kinase activity between nonobese and obese control subjects, although it was significantly lower in obese NIDDM patients (25). Thus, our studies demonstrate in the same population of diabetic patients a functional defect of insulin receptor in the liver and a peripheral target organ of insulin action.

Our data do not define the primary event, i.e., whether defective insulin-receptor kinase causing hyperglycemia in diabetics or diabetes per se is responsible for decreased insulin-receptor kinase activity. Also, because of the limited amount of tissue, we were unable to study insulin-receptor autophosphorylation. Thus, this study does not imply that autophosphorylation of insulin receptor in NIDDM is also defective because Freidenberg et al. (26) showed a dissociation between autophosphorylation and phosphorylation of exogenous substrates. Also, important differences in in vitro and intact cell phosphorylation of insulin receptors have been observed (27). Therefore, our study only supports the hypothesis that the insulin resistance in NIDDM may be at least partially due to a defect in the insulin-receptor kinase activity. It is also possible that some metabolic abnormality associated with NIDDM results in defective tyrosine kinase activity of insulin receptors.

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