Increased Expression of Insulin/Insulin-like Growth Factor-I Hybrid Receptors in Skeletal Muscle of Noninsulin-dependent Diabetes Mellitus Subjects

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Abstract

Insulin receptors (IR) and IGF-I receptors (IGF-IR) have been shown to form hybrid receptors in tissues coexpressing both molecules. To date there is no information about the distribution of hybrids in tissues of normal or diabetic subjects. We developed a microwell-based immunoassay to quantitate hybrids in small human tissues samples. Micro-wells were coated with MA-20 anti-IR antibody or α-IGF-IR—PA antibody directed against the IGF-IR α-subunit, and incubated with skeletal muscle extracts of patients with non-insulin-dependent diabetes mellitus (NIDDM) and normal controls. Immobilized receptors were incubated with 125I-insulin or 125I-IGF-I in the presence or absence of the two unlabeled ligands. Hybrids were quantified as the fraction of 125I-IGF-I binding immunoadsorbed with MA-20 and expressed as percentage of total IGF-IR (type I+ hybrids) immobilized with α-IGF-IR—PA. The immunoassay was validated using Western blotting analysis. Relative abundance of hybrids detected in NIDDM patients was higher than in controls. The percentage of hybrids was negatively correlated with IR number and in vivo insulin sensitivity measured by an insulin tolerance test, whereas the percentage was positively correlated with insulin resistance. Insulin binding affinity was lower in NIDDM patients than in controls, and was correlated with the percentage of hybrids. Maximal IGF-I binding was significantly higher in muscle from NIDDM patients compared to controls and was positively correlated with the percentage of hybrid receptors whereas IGF-I binding affinity did not differ between the two groups. These results raise the possibility that alterations in expression of hybrid receptors may contribute to decreased insulin sensitivity, and to increased sensitivity to IGF-I. Because IGF-I has been proposed as a hypoglycemic agent in NIDDM, these results are relevant to the development of new approaches to the treatment of insulin resistance of NIDDM. (J. Clin. Invest. 1996. 98:2887–2893.) Key words: insulin receptor • IGF-I receptor • hybrid receptor • insulin resistance

Introduction

The pathophysiology of noninsulin-dependent diabetes mellitus (NIDDM) involves defects in both insulin secretion and peripheral insulin action (1). Skeletal muscle is a major site of insulin-stimulated glucose disposal and has been suggested to be the primary tissue responsible for insulin resistance in the postabsorptive state (2). Insulin action is initiated through hormone binding to cell surface receptors which triggers a cascade of intracellular phosphorylation events (3). The insulin receptor is a glycoprotein which shares structural and functional homology with the insulin-like growth factor (IGF-I) receptor (4–6). Both receptors are composed of two α-subunits linked to β-subunits and to each other by disulfide bonds and noncovalent interactions. The α- and β-subunits are synthesized as a single-chain proreceptor which is glycosylated and proteolytically cleaved to yield the mature α2β2 heterotetrameric receptor. The α-subunits are entirely extracellular and contain the hormone binding site(s), while the membrane-spanning β-subunits possess the intrinsic tyrosine kinase activity in their cytoplasmic domain. Several lines of evidence have suggested that hybrid receptors composed of an insulin receptor αβ-heterodimer and an IGF-I αβ-heterodimer are formed in tissues coexpressing the two receptors (7, 8). Hybrid receptors were first identified by studies demonstrating high affinity IGF-I binding immunoreactive with insulin receptor-specific antibodies (7). The formation of hybrid receptors was subsequently shown in a variety of transfected rodent cells overexpressing insulin or IGF-I receptors by examining their reactivity with species- and receptor-specific antibodies (9). Assembly of insulin/IGF-I hybrid receptors was also demonstrated in vitro under defined ligand incubation conditions (10). The functional significance of these hybrid receptors is still undefined. Previous studies have shown that insulin/IGF-I hybrid receptors bind IGF-I with an affinity similar to that seen with type I IGF receptors, but bind insulin with lower affinity than classic insulin receptors (11, 12). Furthermore, hybrid receptors function more like an IGF-I receptor rather than an insulin receptor with respect to receptor autophosphorylation, and IGF-I internalization and degradation (12–14). Therefore, an increased proportion of insulin/IGF-I hybrid receptors would be expected to reduce insulin binding, and, thereby, insulin sensitivity in tissues expressing both receptors. Based on these observations, we asked the question of whether increased expression of insulin/IGF-I hybrid receptors might contribute, at least in part, to the insulin resistance of NIDDM. To this end, we have measured the abundance of insulin/IGF-I hybrid re-
ceptrons in skeletal muscle from normal and NIDDM subjects using a microwell-based immunoassay and correlated hybrid receptors expression with in vivo insulin sensitivity.

Methods

Materials. Tissue samples of subcutaneous rectus abdominus skeletal muscle were obtained during elective abdominal surgery from eight nonobese normal subjects and eight NIDDM patients admitted to the hospital for cholecystectomy or total hysterectomy. Tissue samples were cleaned of all connective tissue and blood, immediately frozen in liquid nitrogen, and stored at −70°C until use. Clinical and biochemical data of the subjects are shown in Table I. The control subjects had normal fasting plasma glucose levels, normal blood pressure, and no family history of diabetes. All NIDDM patients were diagnosed according to the criteria of the National Diabetes Data Group (15), and were treated with diet alone. No subject had taken any other medication known to alter insulin or glucose metabolism. Insulin sensitivity was assessed by i.v. insulin tolerance test (ITT), performed as previously described (16). After an overnight fast, a single bolus of regular insulin (0.1 U/kg body wt i.v.) was injected into an antecubital vein. Blood samples were collected at 15 and 5 min before and 3, 6, 9, 12, 15, 20, and 30 min after insulin injection. Glucose was injected at the end of ITT to stop the fall in plasma glucose. The constant rate for plasma glucose disappearance (Kg) was calculated according to the formula 0.693/t½. The plasma glucose t½ was calculated from the slope of least square analysis of the plasma glucose concentrations from 3–15 min after intravenous insulin injection, when the plasma glucose concentration declined linearly. Consent was obtained from all subjects after the nature of the procedure was explained. The study was performed in accordance with the principles of the Declaration of Helsinki.

A polyclonal antibody (α-IGF-I receptor [R]–PA) was raised in rabbit against a synthetic peptide corresponding to residues 642–661 of the IGF-I receptor α-subunit sequence (6), according to previously described method (17). MA-20 monoclonal antibody to the insulin receptor was produced as previously described (18).

Human 125I-A14-monoiiodoinsulin (290–320 μCi/μg) was a gift from Dr. Luca Benzi (University of Pisa, Pisa, Italy) (19). 125I-IGF-I (280–310 μCi/μg) was purchased from DuPont-New England Nuclear (Wilmington, DE). Human recombinant insulin was graciously provided by Novo-Nordisk A/S (Bagsværd, Denmark). Human recombinant IGF-I was obtained from Boehringer Mannheim Biochemica (Mannheim, Germany). All other chemicals were from Sigma Chemicals Co. (St. Louis, MO).

Tissue solubilization. Extracts from skeletal muscle were prepared by solubilization in 50 mM Heps buffer, pH 7.6, containing 150 mM NaCl, 1% Triton X-100, 1 mg/ml bacitracin, 2 mM PMSF, 1,000 U/ml aprotinin, 0.1% BSA, and incubated with tissue extracts (500 μg in 40 μl) for 16 h at 4°C. The wells were then washed three times with binding buffer and immobilized receptors were incubated in the same buffer with 125I-IGF-I (60 PM) or 125I-insulin (60 PM) for 16 h at 4°C in the presence or absence of various concentrations of the corresponding unlabelled ligands. Thereafter, the wells were washed three times with binding buffer to remove unbound ligands. Radioactivity bound to immobilized receptors was collected by adding 2% SDS for 30 min at 24°C to the wells, and counted in a gamma counter.

Insulin and IGF-I binding data were analyzed by the method of Scatchard (21) using the LIGAND computer program.

Western blotting. Equal amount of tissue extracts (500 μg) were incubated for 16 h at 4°C with 1 μg of either α-IGF-IR–PA or MA-20 antibody each bound to Protein A-Sepharose. The immunoprecipitates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. Proteins (500 μg/lane) resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose filters. The nonspecific binding sites of membranes were blocked by a 2-h incubation of Tris-buffered saline (TBS, pH 7.5) containing 5% BSA. The filters were then incubated for 16 h at 4°C with affinity-purified α-IGF-IR-PA antipeptide antibody (1:100 dilution). After extensive washings with TBS, the bound antibody was detected with alkaline phosphatase-conjugated anti-rabbit immunoglobulins. Band densities were quantified by bidimensional densitometry using a scanning densitometer (model GS670; Bio-Rad Laboratories, Richmond, CA).

Statistical analysis. Unpaired Student’s t test was used to compare mean values. Linear correlations between variables were tested by calculating Pearson’s correlation coefficient.

Results

Characterization of microwell-based immunoassay. To measure insulin and IGF-I binding to receptors and quantitate the abundance of insulin/IGF-I hybrid receptors, receptors were immunoadsorbed on a microwell plate using MA-20, an anti-insulin receptor monoclonal antibody that does not cross-react with IGF-I receptors or α-IGF-IR–PA, an anti–IGF-I receptor polyclonal antibody that does not cross-react with insulin receptors (18, 22). Microwells coated with either MA-20 or α-IGF-IR–PA antibody were incubated with muscle extracts, and ligand binding characteristics of immobilized receptors were examined by competition–inhibition binding studies. Binding of insulin and IGF-I to their own receptors isolated from muscle extracts was similar to that obtained by immunoprecipitation of solubilized receptors. Furthermore, the immunoassay with either antibody was quantitative since both α-IGF-IR–PA and MA-20 were able to remove >92% of their respective receptors in immunodepletion experiments.

Ligand binding studies in skeletal muscle of normal and NIDDM subjects. Tissue extracts were prepared from muscle samples of NIDDM patients and normal subjects (n = 8 each) and added to microwell coated with MA-20 or α-IGF-IR–PA antibody. After washing, ligand binding to immobilized receptors was assessed by incubating the wells with 125I-labeled insulin or IGF-I in the presence or absence of varying concentrations of unlabelled ligand. Maximal insulin binding to MA-20–immobilized receptors was significantly lower in muscle from NIDDM patients compared to normal individuals (B/T =

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Table I. Clinical Characteristics of Study Subjects

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<thead>
<tr>
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<th>Normal</th>
<th>NIDDM</th>
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<tr>
<td>Sex (M/F)</td>
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<td>4/4</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>50±6</td>
<td>61±4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.9±1</td>
<td>26.2±1</td>
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<tr>
<td>Fasting glucose (mmol/liter)</td>
<td>3.6±0.7</td>
<td>4.2±0.8</td>
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<tr>
<td>Fasting insulin (μU/ml)</td>
<td>6.0±2.4*</td>
<td>10.2±3.0*</td>
</tr>
<tr>
<td>Insulin tolerance (Km)</td>
<td>3.9±0.6³</td>
<td>1.8±0.6³</td>
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*P < 0.0012; †P < 0.0001.
1.40±0.2 and 3.02±0.5; \( P < 0.003 \), respectively). The mean values for total \(^{125}\text{I}\)-insulin binding were 6,040±1,200 cpm/mg protein for normal subjects vs. 2,800±420 cpm/mg for NIDDM patients. Binding inhibition curves for the two groups of subjects are shown in Fig. 1. Receptor binding affinity, estimated as the concentration of unlabeled insulin required to inhibit 50% of maximal \(^{125}\text{I}\)-insulin binding to MA-20–immobilized receptors (IC\(_{50}\)), was significantly decreased in muscle from patients with NIDDM.

**Figure 1.** Inhibition of \(^{125}\text{I}\)-insulin or \(^{125}\text{I}\)-IGF-I binding to immobilized receptors. Tissue extracts prepared from skeletal muscle of eight NIDDM patients (open circles) and eight normal subjects (closed squares) were used to measure insulin (inset, A) or IGF-I (inset, B) binding to immobilized receptors as described in Methods. Results of insulin (A) or IGF-I (B) binding competition expressed as percent of maximal binding are presented as mean±SEM. Scatchard plots of insulin (inset, A) and IGF-I (inset, B) binding data.
NIDDM patients compared to normal subjects (IC_{50} = 1.29±0.13 and 0.47±0.1 nM; P < 0.007, respectively). Scatchard analysis of insulin binding data showed that the number and affinity of the binding sites were reduced in NIDDM patients compared to normal subjects (Ro = 14.5±1.2 pmol/mg protein; K_d = 0.13±0.01 nM and Ro = 9.44±0.9 pmol/mg protein; K_d = 0.52±0.02 nM for normal subjects and NIDDM patients, respectively) (inset, Fig. 1 A). The number of insulin receptors was inversely correlated with fasting insulin levels (r = −0.60; P < 0.02). Maximal IGF-I binding to α-IGF-IR-PA-immobilized receptors was significantly higher in muscle from NIDDM patients compared to normal individuals (B/T = 1.8±0.36 and 0.85±0.27; P < 0.05, respectively). The mean values for total {\textsuperscript{125}}I-IGF-I binding were 1,900±440 cpm/mg protein for NIDDM patients versus 800±170 cpm/mg for normal subjects. As shown in Fig. 1, receptor binding affinity was similar in the two groups of subjects (IC_{50} = 0.36±0.1 and 0.44±0.09 nM, respectively, in NIDDM patients and normal subjects). Scatchard analysis of IGF-I binding data revealed that an increased number of binding sites in NIDDM patients compared to normal subjects (Ro = 7.9±0.7 pmol/mg protein and Ro = 3.25±0.3 pmol/mg protein, respectively) whereas binding affinity was similar in the two groups (K_d = 0.6±0.2 nM and 0.9±0.2 nM for NIDDM patients and normal subjects, respectively) (inset, Fig. 1 B).

**Quantitation of insulin/IGF-I hybrid receptors in skeletal muscle of normal and NIDDM subjects.** The assay of insulin/IGF-I hybrid receptors was based on their ability to bind IGF-I, but not for insulin, with high affinity, and to react with specific antiinsulin receptor antibody (7, 9, 11). Tissue extracts obtained from skeletal muscle of NIDDM patients and normal subjects (n = 8 each) were added to microwell coated with MA-20 antiinsulin receptor antibody. MA-20–immobilized receptors were then incubated with {\textsuperscript{125}}I-IGF-I (60 pM) in the presence or absence of increasing amounts of unlabeled IGF-I and hybrid receptors were estimated as the fraction of {\textsuperscript{125}}I-IGF-I binding immunoadsorbed with MA-20 antibody vs. total of {\textsuperscript{125}}I-IGF-I binding sites (hybrids + type I receptors) immunoadsorbed with α-IGF-IR–PA antibody. Under these conditions, no significant {\textsuperscript{125}}I-IGF-I binding to insulin receptors was detected, as deduced by the inability of low concentration of unlabeled insulin to compete for {\textsuperscript{125}}I-IGF-I binding to MA-20–immobilized receptors (IC_{50} values for competition by IGF-I of 0.25 nM and by insulin of 107 nM). As shown in Fig. 2, the percentage of insulin/IGF-I hybrid receptors was significantly higher in muscle from NIDDM patients compared to normal subjects (74.0±9.0 vs. 44.3±1.6%, respectively, P < 0.0001). To further validate the microwell-based immunoassay, quantitation of hybrid receptors was assessed by precipitating receptors from muscle extracts with α-IGF-IR–PA or MA-20 antibody, and immunoblotting with α-IGF-IR–PA antibody. A representative Western blot is shown in Fig. 3, in which α-IGF-IR–PA and MA-20 antibody precipitate a single molecular species of approximately M_{r} 130,000 D, corresponding to the α subunit of the IGF-I receptor. Scanning densitometry was used to quantitate the levels of the M_{r} 130,000 D protein precipitated by MA-20 antibody and immunoblotted with α-IGF-IR–PA antibody. Mean levels of hybrid receptors were 1.6-fold

**Figure 2.** Quantitation of insulin/IGF-I hybrid receptors in skeletal muscle of normal and NIDDM subjects. Tissue extracts were added to microwell coated with MA-20 or α-IGF-IR–PA antibody, and immobilized receptors were then incubated with {\textsuperscript{125}}I-IGF-I (60 pM) in the presence or absence of unlabeled IGF-I (100 nM). Hybrid receptors were estimated as the percentage of {\textsuperscript{125}}I-IGF-I binding sites reactive with MA-20 antibody vs. total of {\textsuperscript{125}}I-IGF-I binding sites (hybrids + type I receptors) reactive with α-IGF-IR–PA antibody. Each point represents the mean value for a single subject measured in three experiments each in triplicate. The mean for the two groups of subjects is shown as a horizontal bar.

**Figure 3.** Quantitation of insulin/IGF-I hybrid receptors by Western blotting. Equal amount of tissue extracts (500 μg) prepared from skeletal muscle of two NIDDM patients (lanes 1 and 2) and two normal subjects (lanes 3 and 4) were precipitated with α-IGF-IR–PA or MA-20 antibody. The immunoprecipitates were subjected to SDS–PAGE under reducing conditions. Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose filters. The filters were incubated with affinity-purified α-IGF-IR–PA antibody (1:100 dilution), and the bound antibody was detected with alkaline phosphatase-conjugated anti–rabbit immunoglobulins. Band densities were quantified by scanning densitometry. The intensities expressed as arbitrary densitometric units were: 3.42 (lane 1 a), 3.52 (lane 2 a), 2.51 (lane 1 b), 2.09 (lane 2 b), 1.28 (lane 3 a), 1.04 (lane 4 a), 0.53 (lane 3 b), and 0.45 (lane 4 b).
higher in NIDDM patients as compared with normal subjects \((P < 0.001)\). These results were in close agreement with those obtained with the microwell-based immunoassay. The percentage of hybrid receptors was positively correlated with fasting insulin levels \((r = 0.75; P < 0.0008)\), with IGF-I receptor number \((r = 0.63; P < 0.008)\), and IC\(_{50}\) of insulin binding inhibition \((r = 0.60; P < 0.02)\), whereas the percentage was negatively correlated with insulin receptor number \((r = -0.60; P < 0.01)\) and in vivo insulin sensitivity measured by ITT \((r = -0.7, P < 0.005)\; \text{Fig. 4})

**Discussion**

The molecular mechanism(s) responsible for insulin resistance in NIDDM is still unclear. In the past 20 yr, investigators have extensively studied the role of insulin receptors in the patho-

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**Figure 4.** Relative abundance of hybrid receptors in skeletal muscle from NIDDM patients (open symbols) and normal subjects (closed symbols) in relation to fasting insulin levels (A), maximal IGF-I binding (B), IC\(_{50}\) of insulin binding inhibition (C), maximal insulin binding (D), and in vivo insulin sensitivity (\(K_{itt}\)) (E).

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*Insulin/IGF-I Hybrid Receptors in Muscle of NIDDM Subjects* 2891
 genesis of insulin resistance. Recently, it has become apparent that an insulin receptor α–β-hemireceptor and an IGF-I receptor α–β-hemireceptor assemble as hybrid receptors in cells and tissues expressing both receptors (7–14). A limited functional characterization of hybrid receptors has been performed in studies of cells overexpressing insulin receptors and with affinity-purified hybrid receptors (11–14). These studies indicate that hybrid receptors are functionally similar to type-I IGF receptors, rather than insulin receptors, with respect to hormone binding affinity, receptor autophosphorylation, ligand internalization, and degradation. Thus, the presence of hybrid receptors would be expected to affect insulin binding affinity and decrease insulin sensitivity in tissues expressing both receptors. It is possible that increased expression of hybrid receptors in target tissues of insulin action contributes to insulin resistance. In the present study, we have investigated the relative distribution of hybrid receptors in skeletal muscle of normal and NIDDM subjects. We have developed and applied a novel microwell-based immunoassay that is capable of quantitating the relative abundance of hybrid insulin/IGF-I receptors in small samples of human tissue. Quantitation of hybrid receptors revealed that the proportion of hybrids was significantly higher in skeletal muscle of NIDDM patients compared to normal subjects. The proportion of hybrid receptors was correlated with a decrease in both insulin binding affinity and in vivo insulin sensitivity measured by an insulin tolerance test. These data indicate that alterations in the relative abundance of insulin/IGF-I hybrid receptors in skeletal muscle may be associated with reduced insulin sensitivity in NIDDM patients. Thus far, there have been no reports of decreased insulin binding affinity in skeletal muscle of NIDDM patients (23, 24). The reason for this discrepancy is unclear, but it may be due to methodological differences. In the present study, measurements of insulin binding were performed with highly enriched immobilized insulin receptors, which include insulin holoreceptors and hybrid receptors. In contrast, in previous studies insulin binding assays were performed on partially purified lectin preparations which include insulin, hybrid, and type-I IGF receptors. Thus, copurification of type-I IGF receptors with insulin receptors could explain the differences between our results and those of previous studies. Alternative explanations include contamination of muscle specimens by other tissues, differences in clinical characteristics of the subjects examined, or other confounding variables.

It is not known how the expression of insulin/IGF-I hybrid receptors is regulated. Hybrids are presumably formed in the endoplasmic reticulum by disulfide linkage of proreceptors at an early posttranslational step. Our results raise the possibility that increased expression of IGF-I receptors and/or decreased expression of insulin receptors could increase the number of hybrid receptors. Studies with transfected rodent cells expressing the human insulin receptor (14) or coexpressing both the insulin receptor and the IGF-I receptor (13) have led to similar conclusions. Taken together, these results suggest that the proportion of hybrid receptors in tissues expressing both receptors may be a function of the relative number of insulin and IGF-I receptors. The observed decrease in insulin receptor expression in NIDDM is consistent with previous studies showing a significant decrease in insulin binding to skeletal muscle of NIDDM patients and could be a consequence of elevated plasma insulin levels (23). In the current study, plasma insulin levels were significantly higher in NIDDM patients than in normal subjects, and were correlated with a decreased number of insulin receptors and an increased proportion in insulin/IGF-I hybrid receptors. Alternative explanations are also possible. For example, alterations in muscle fiber type composition might explain the NIDDM-associated decreased expression of the insulin receptor and increased expression of hybrid receptors. It has been demonstrated in rats that insulin binding is higher in type I, slow twitch oxidative fibers than in type IIb, fast twitch glycolytic fibers (25). Therefore, lower levels of slow twitch oxidative fibers in NIDDM patients may result in a decreased number of insulin receptors and an increased number of hybrid receptors. However, this possibility seems unlikely because several previous studies in humans failed to detect differences in fiber type composition between normal and NIDDM subjects (26–28). It is worthy to note that the decrease in insulin-receptor expression in NIDDM does not reflect a generalized decrease in all muscle proteins, because IGF-I receptor's number was increased, and the estimated recovery of total muscle proteins did not differ between the two groups. Alternatively, increased expression of insulin/IGF-I hybrid receptors may represent a primary defect rather than a secondary event due to hyperinsulinemia. Further studies are required to address this issue.

Recombinant human IGF-I (rhIGF-I) has been proposed as a potential therapy for NIDDM (29–31). Preliminary studies have shown that treatment with rhIGF-I increases insulin sensitivity and improves glycemic control in NIDDM patients (30, 32). The mechanisms by which IGF-I exerts these effects in vivo are unclear as IGF-I can act through type-I IGF receptors, insulin receptors, or both. Our data raise the intriguing possibility that rhIGF-I might stimulate glucose transport and glycogen synthesis in muscle through insulin/IGF-I hybrid receptors. Therefore, increased expression of hybrid receptors that bind IGF-I with higher affinity than insulin may contribute to decrease insulin sensitivity, and increase sensitivity to rhIGF-I in NIDDM patients. Alternatively, rhIGF-I may enhance insulin sensitivity by downregulating type I IGF receptors thus decreasing insulin/IGF-I hybrid receptor content.

In summary, we developed a novel microwell-based immunoassay to assess the distribution of insulin/IGF-I hybrid receptors in human tissues. Using this method, we have provided the first direct evidence for increased expression of insulin/IGF-I hybrid receptors in NIDDM patients as compared to normal subjects. Increased expression of hybrid receptors was correlated with a decrease in both insulin binding affinity and in vivo insulin sensitivity measured by insulin tolerance test. These results raise the possibility that alterations in expression of hybrid receptors may contribute, at least in part, to insulin resistance, a major component of NIDDM.

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Federici et al. 2892


**Insulin/IGF-I Hybrid Receptors in Muscle of NIDDM Subjects**