Expression of Insulin/IGF-I Hybrid Receptors Is Increased in Skeletal Muscle of Patients With Chronic Primary Hyperinsulinemia

Massimo Federici, Davide Lauro, Monica D'Adamo, Barbara Giovannone, Ottavia Porzio, Monica Mellozzi, Guido Tamburrano, Paolo Sbraccia, and Giorgio Sesti

The insulin receptor (IR) shares structural and functional homology with the IGF-I receptor (IGF-IR). Hybrid receptors composed of an IR αβ-heterodimer and an IGF-IR αβ-heterodimer are formed in tissues expressing both molecules. Hybrids behave as IGF-IR rather than IR with respect to ligand binding affinity, receptor autophosphorylation, and hormone internalization and degradation. Factors regulating hybrid formation in vivo are unknown. We recently reported that in skeletal muscle of NIDDM patients, expression of hybrids is increased and correlated with a decrease in IR number and an increase in fasting insulin levels. However, it is not clear whether increased expression of hybrid receptors is a primary defect specifically associated with NIDDM or a secondary event caused by hyperinsulinemia. To address this issue, we used a quantitative microwell-based immunoassay to measure hybrid receptor abundance in skeletal muscle of 11 normal subjects and 12 patients with insulinoma, a state of primary nongenetically determined hyperinsulinemia. Total insulin binding was lower in insulinoma patients than in normal subjects (0.70 ± 0.18 vs. 4.59 ± 0.77; $P < 0.0001$). Total IGF-I binding did not differ between the two groups (0.81 ± 0.27 and 0.85 ± 0.10, respectively). The amount of hybrids, expressed as bound/total (B/T), was higher in patients with insulinoma than in normal subjects (0.57 ± 0.19 vs. 0.36 ± 0.03; $P < 0.0006$) and was inversely correlated with total insulin binding ($r = -0.64$, $P < 0.0004$). Increased abundance of hybrid receptors was positively correlated with insulin levels ($r = -0.82$, $P < 0.0009$) and inversely correlated with insulin-mediated glucose uptake ($r = -0.80$, $P < 0.01$). No correlations were observed between insulin-mediated glucose uptake and maximal specific insulin binding ($r = 0.19$, $P = 0.64$). These results indicate that insulin-induced IR downregulation may lead to the formation of a higher proportion of hybrid receptors, whose abundance is negatively correlated with in vivo insulin sensitivity. These results, therefore, support a role for insulin in the regulation of hybrid receptors formation and suggest that increased expression of hybrids in NIDDM may be a secondary event caused by hyperinsulinemia rather than a primary defect. *Diabetes* 47:87–92, 1998

Insulin and IGF-I exert their biological actions by interacting with transmembrane tyrosine-kinase receptors that share structural and functional homology (1–3). Both the insulin receptor and the IGF-I receptor (IGF-IR) are composed of two α-subunits linked to β-subunits and to each other by disulfide bonds. The α-subunits are entirely extracellular and contain the hormone binding site(s), whereas the membrane-spanning β-subunits possess the intrinsic tyrosine kinase activity in their cytoplasmic domain. In tissues co-expressing both receptor genes, hybrid receptors composed of an insulin receptor αβ-hemireceptor and an IGF-IR αβ-hemireceptor are formed (4–8). The physiopathological function of these hybrid receptors is still undefined. Previous studies have shown that hybrid receptors bind IGF-I with an affinity similar to that observed with IGF-IRs, but bind insulin with markedly decreased affinity (9–11). Moreover, hybrid receptors behave as IGF-IRs, rather than as insulin receptors, with respect to receptor autophosphorylation and hormone internalization and degradation (9,12). We recently reported a significant increase in hybrid receptor expression in skeletal muscle of NIDDM patients compared with normal subjects (13). Interestingly, the increased expression of hybrid receptors was correlated with a decrease in insulin receptor number and an increase in fasting insulin levels. Although these data suggest that plasma insulin levels may play a major role in regulating hybrid receptor formation in vivo, it is not clear whether alteration in hybrid receptors expression is a primary defect specifically associated with NIDDM or a secondary event caused by hyperinsulinemia. To address this issue, we used a previously validated microwell-based immunoassay to measure abundance of hybrid receptors in skeletal muscle of patients with insulinoma, a state of primary nongenetically determined hyperinsulinemia, and correlated hybrid receptor abundance with in vivo insulin action.

RESEARCH DESIGN AND METHODS

Materials. Human ^{125}I-labeled A14-monoiodoinsulin (290–320 μCi/μg) and ^{125}I-labeled IGF-I (280–310 μCi/μg) were purchased from Amersham (Buckinghamshire, U.K.). Human insulin was provided by Novo-Nordisk A/S (Bagsværd, Denmark). Recombinant human IGF-I was purchased from Boehringer Mannheim (Mannheim, Germany). The polyclonal antibody α-IGF-IR-PA, an anti-IGF-IR that does not cross-react with insulin receptors (14), was raised in rabbit against a synthetic peptide corresponding to residues 642–661 of the IGF-IR

From the Laboratory of Molecular Medicine (M.F., D.L., B.G., O.P., G.S.), Department of Internal Medicine, and the Division of Endocrinology (M.D., M.M., G.T., P.S.), University of Rome, Rome, Italy.

Address correspondence to Dr. Giorgio Sesti, Dipartimento di Medicina Interna, Università di Roma "Tor Vergata," Via Orazio Raimondo, 00173 Rome, Italy. E-mail: sesti@utovrm.it.

Received for publication 9 May 1997 and accepted in revised form 19 September 1997.

IGF-IR, IGF-I receptor; IGF-IR-PA, IGF-IR polyclonal antibody; IGT, impaired glucose tolerance; IR, insulin receptor.

α-subunit sequence (3), according to the previously described method (15). MA-20 anti-insulin receptor monoclonal antibodies that do not cross-react with IGF-IRs (16) were purchased from Amersham. All other chemicals were from Sigma (St. Louis, MO).

Subjects. We compared 12 patients with surgically proven insulinoma located within the pancreas with 11 nonobese normal subjects. Clinical and biochemical data of the subjects are shown in Table 1. In insulinoma patients, fasting plasma glucose and insulin concentrations were measured both at 8:00 A.M. on 4 different days during the 2 weeks preceding surgery and at the time of muscle biopsy (averages of these values are given in Table 1). The control subjects had normal fasting plasma glucose levels, normal blood pressure, and no family history of diabetes. None of the subjects had other concomitant diseases or had taken any medications known to alter carbohydrate metabolism. Tissue samples of rectus abdominus skeletal muscle were obtained during tumor removal in insulinoma patients or elective abdominal surgery (cholecystectomy or total hysterectomy) in normal subjects. Tissue samples were cleaned of all connective tissue and blood, immediately frozen in liquid nitrogen, and stored at −70°C until use. Consent was obtained from all subjects after the nature and potential risks of the study had been explained. The study was performed in accordance with the principles of the Declaration of Helsinki.

In vivo assessment of insulin action. Insulin-mediated whole-body glucose utilization was determined in 8 of the 14 patients with insulinoma by the hyperinsulinemic-euglycemic clamp technique, as previously described (17). Studies were started at 8:00 A.M. after a 10-h overnight fast. Euglycemia was slowly reached in patients with insulinoma with a constant glucose infusion at a rate of 10 μmol · kg^{-1} · min^{-1}; insulin was then constantly infused at a rate of 1.0 mU · kg^{-1} · min^{-1} for 2 h after 10 min of priming (30 mU/kg). Euglycemia was maintained by a variable glucose infusion using the negative feedback principle. Rates of insulin-mediated glucose uptake were calculated on the basis of glucose infusion rates during the last 60 min of the clamp, when steady state was reached. Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman, Palo Alto, CA) and plasma insulin by double-antibody radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden).

Tissue solubilization. Extracts from skeletal muscle were prepared by solubilization in 50 mmol/l HEPES buffer (pH 7.6) containing 150 mmol/l NaCl, 1% Triton X-100, 1 mg/ml bacitracin, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), and 1,000 units/ml aprotinin for 60 min at 4°C. Insoluble material was removed by centrifugation at 100,000*g* for 60 min at 4°C. Soluble fractions were diluted to 0.2% Triton X-100 and immediately assayed. Protein content of tissue extracts was determined by the Bradford method (18).

Microwell immunoassay. The assay was performed as previously described (13). Briefly, 96-well microwells were coated with α-IGF-IR-PA or MA-20 antibody in 20 mmol/l $NaHCO_3$ (pH 9.6) and incubated for 16 h at 4°C. The wells were washed three times with buffer A containing 50 mmol/l HEPES buffer (pH 7.6), 150 mmol/l NaCl, 0.1% Triton X-100, 1 mg/ml bacitracin, 2 mmol/l PMSF, 1,000 units/ml aprotinin, and 0.1% bovine serum albumin and incubated with tissue extracts (500 μg) for 16 h at 4°C. The wells were then washed three times with buffer A, and immunoadsorbed receptors were incubated with ^{125}I-labeled IGF-I (60 pmol/l) or ^{125}I-labeled insulin (60 pmol/l) for 16 h at 4°C in the presence or absence of various concentrations of unlabeled ligands. Thereafter, the wells were washed three times to remove unbound ligands. Radioactivity bound to immunoadsorbed receptors was collected by adding 2% SDS for 30 min at 24°C to the wells, and counted in a gamma counter.

Statistical analysis. Results are presented as means ± SE. An 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. A microwell-based immunoassay was used to measure insulin and IGF-I binding to receptors and to quantitate the abundance of insulin/IGF-I hybrid receptors. To make the assay quantitative under conditions of antibody excess, titration of the amount of antibodies bound to wells was performed by immunodepletion experiments. Microwells were coated with increasing concentrations of either MA-20 or α-IGF-IR-PA and incubated with a fixed amount of muscle extracts (500 μg/well). After removal of supernatants, insulin or IGF-I binding to immobilized receptors was determined by incubating the wells with ^{125}I-insulin or ^{125}I-IGF-I in the presence or absence of unlabeled ligands. Residual insulin or IGF-I binding activity in the supernatants was assayed by both precipitation with PEG 6000 and immunoprecipitation with MA-20 or α-IGF-IR-PA bound to protein A-sepharose beads. As shown in Fig. 1, MA-20 was able to remove insulin binding activity in a dosage-dependent manner, as assayed by immunoprecipitation, with the maximal effect (>91% immunodepletion) occurring at 10 μg/well, with no further increase at higher antibody concentrations. Similarly, α-IGF-IR-PA removed IGF-I binding activity in a dosage-dependent manner, with the maximal effect (>93% immunodepletion) occurring at the same concentration required for MA-20 maximal effect (10 μg/well). Similar results were obtained when residual insulin or IGF-I binding activity in the supernatants were determined by the PEG precipitation method (data not shown). For hybrid receptors, immunodepletion experiments were performed by incubating microwells coated with increasing concentrations of MA-20 with a fixed amount of muscle extracts (500 μg/well). After removal of supernatants, ^{125}I-IGF-I binding to immobilized receptors was determined as described above, whereas residual IGF-I binding activity in the supernatants was assayed by immunoprecipitation with MA-20 bound to protein A-sepharose beads. As shown in Fig. 1, MA-20 immunodepleted hybrid receptors in a dosage-dependent manner, with the maximal effect (>90% immunodepletion) occurring at the same concentration required to remove insulin binding activity (10 μg/well). Therefore, subsequent assays to determine insulin, IGF-I, and hybrid receptor abundance were performed under conditions of antibody excess by using microwells coated with 10 μg/well of MA-20 or α-IGF-IR-PA.

TABLE 1
Clinical characteristics of study subjects

	Normal subjects	Insulinoma patients
Sex (M/F)	5/6	5/7
Age (years)	58 ± 8	47 ± 6
BMI (kg/m^2)	25.1 ± 2.1	28.9 ± 5.1
Fasting plasma glucose (mmol/l)	4.93 ± 0.5*	2.6 ± 0.2*
Fasting plasma insulin (μU/ml)	8.4 ± 0.9‡	60.5 ± 12.2‡

Data are means ± SE. *P < 0.0001; ‡P < 0.0009.

Insulin and IGF-I binding studies in skeletal muscle of normal subjects and insulinoma patients. Muscle extracts from normal subjects and insulinoma patients were added to an MA-20–coated microwell, and insulin binding to immobilized receptors was examined by competition-inhibition binding studies. As shown in Fig. 2*A*, receptor binding affinity, estimated as the concentration of unlabeled insulin required for half-maximal inhibition of ^{125}I-insulin binding to immobilized receptors (IC_{50}), was similar in the two groups of subjects studied (0.57 ± 0.09 and 0.59 ± 0.05 nmol/l insulin, normal subjects and insulinoma patients, respectively). Maximal specific insulin binding to MA-20–immobilized receptors was significantly higher in muscle from normal subjects than from insulinoma patients (4.59 ± 0.77 vs. 0.70 ± 0.18; P < 0.0001). The mean values for maximal specific ^{125}I-insulin binding were 4,590 ± 770 cpm/mg protein for normal subjects vs. 700 ± 180 cpm/mg for insulinoma patients. Nonspecific ^{125}I-

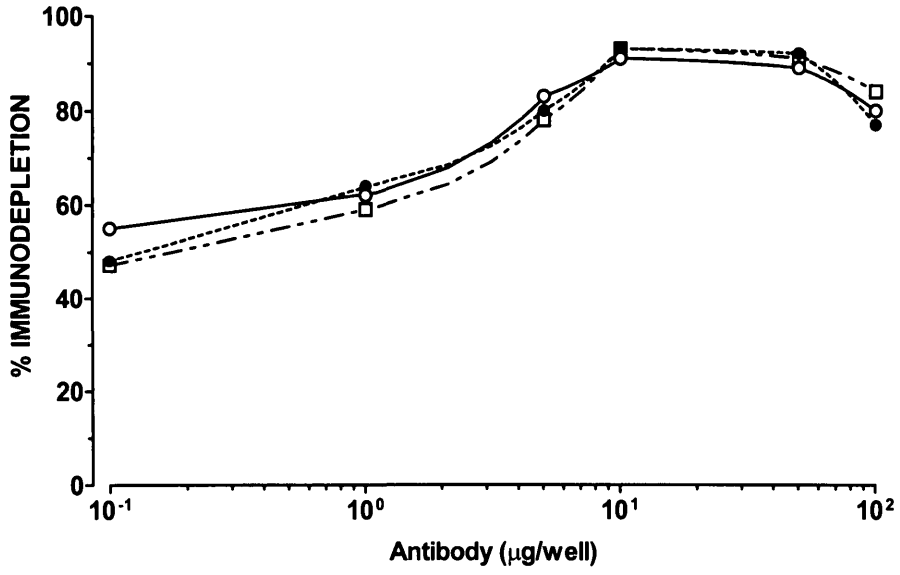

FIG. 1. Immunodepletion of insulin (○), IGF-I (●), and hybrid receptors (□). Microwells were coated with increasing concentrations of either MA-20 (○, □) or α-IGF-IR-PA (●), and incubated with a fixed amount of muscle extracts (500 μg/well). After removal of supernatants, insulin or IGF-I binding to immobilized receptors was determined by incubating the wells with ^{125}I-insulin (○) or ^{125}I-IGF-I (□, ●) in the presence or absence of unlabeled ligands. Residual insulin or IGF-I binding activity in the supernatants was assayed by immunoprecipitation with MA-20 (○, □) or α-IGF-IR-PA (●) bound to protein A-sepharose beads. A representative experiment of three experiments performed in triplicate is shown. Irrelevant monoclonal antibody did not deplete any receptor.

insulin binding determined in the presence of 1 μmol/l unlabeled insulin was 8 ± 3% of total ^{125}I-insulin binding. Insulin levels were higher in insulinoma patients than in control subjects and were inversely correlated with maximal specific insulin binding (r = –0.63, P < 0.002).

IGF-I binding studies were performed by adding muscle extracts from normal subjects and insulinoma patients to a microwell coated with α-IGF-IR-PA antibody, and incubating the immobilized receptors with ^{125}I-IGF-I in the presence or absence of unlabeled IGF-I. As shown in Fig. 2*B*, receptor affinity for IGF-I binding was similar in the two groups of subjects (IC_{50} = 0.56 ± 0.07 and 0.59 ± 0.09 nmol/l IGF-I, for normal subjects and insulinoma patients, respectively). Maximal specific ^{125}I-IGF-I binding to α-IGF-IR-PA–immobilized receptors did not differ between the two groups (0.85 ± 0.10 and 0.81 ± 0.27, for normal subjects and insulinoma patients, respectively). The mean values for maximal specific ^{125}I-IGF-I binding were 850 ± 100 cpm/mg protein for normal subjects vs. 810 ± 270 cpm/mg for insulinoma patients. Nonspecific ^{125}I-IGF-I binding determined in the presence of 1 μmol/l unlabeled IGF-I was 9 ± 4% of total ^{125}I-IGF-I binding.

Quantitation of insulin/IGF-I hybrid receptors in skeletal muscle of normal subjects and insulinoma patients. The assay was based on the ability of hybrid receptors to bind IGF-I with high affinity and interact with specific anti-insulin receptor antibody (7,9,13). Microwells coated with MA-20 antibody were incubated with muscle extracts from normal subjects and insulinoma patients. After washing, MA-20–immobilized receptors were incubated with ^{125}I-IGF-I in the presence or absence of unlabeled IGF-I or insulin. Figure 3 shows representative competition-inhibition curves of ^{125}I-IGF-I binding to immobilized hybrid receptors. In both groups of subjects, binding of ^{125}I-IGF-I to hybrid receptors was inhibited by low concentrations of unlabeled IGF-I, similar to those observed with IGF-IRs (Fig. 2*B*). In contrast, low concentrations of unlabeled insulin were unable to compete

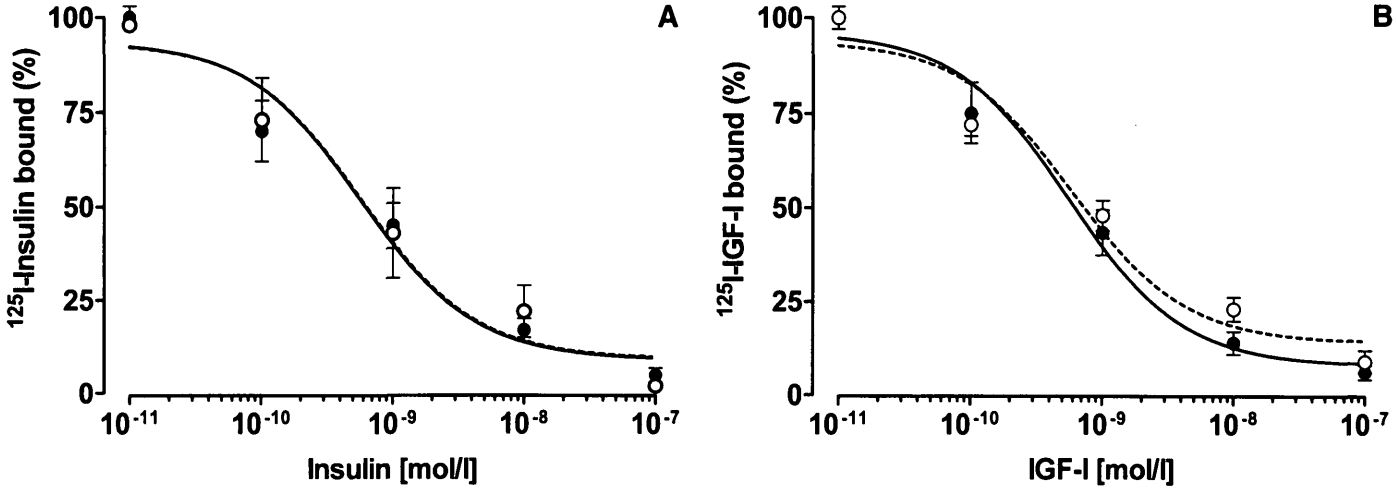

FIG. 2. Inhibition of ^{125}I-insulin or ^{125}I-IGF-I binding to immobilized receptors. Muscle extracts from 12 insulinoma patients (○) and 11 control subjects (●) were added to microwells coated with MA-20 (*A*) or α-IGF-IR-PA (*B*). After washing, ligand binding to immobilized receptors was assessed by incubating the wells with ^{125}I-insulin (*A*) or ^{125}I-IGF-I (*B*) in the presence or absence of increasing concentrations of unlabeled ligands. Results of insulin (*A*) or IGF-I (*B*) binding competition expressed as the percent of maximal specific binding are presented as means ± SE of three experiments carried out in triplicate.

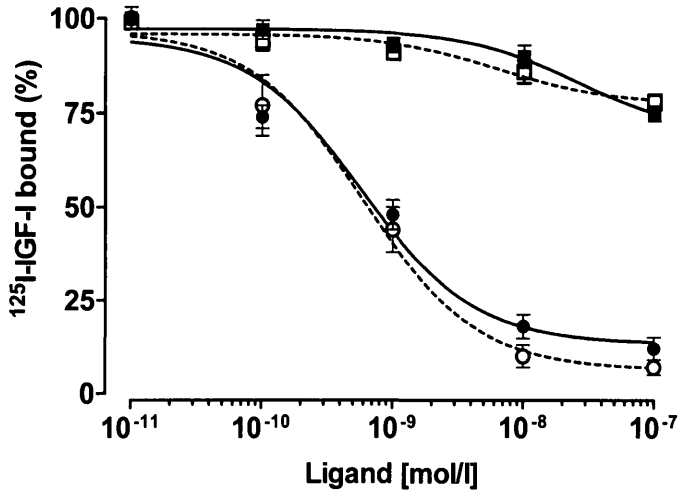

FIG. 3. Inhibition of ^{125}I-IGF-I binding to immunoadsorbed hybrid receptors. Muscle extracts from 12 insulinoma patients (○, □) and 11 control subjects (●, ■) were added to microwells coated with MA-20 anti-insulin receptor antibody. After washing, ligand binding to immobilized receptors was assessed by incubating the wells with ^{125}I-IGF-I in the presence or absence of increasing concentrations of unlabeled IGF-I (○, ●) or insulin (□, ■). Results of IGF-I binding competition expressed as the percent of maximal specific binding are presented as means ± SE of three experiments carried out in triplicate.

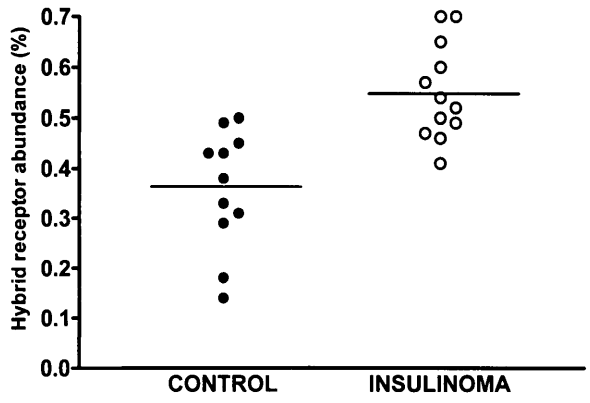

FIG. 4. Quantitation of insulin/IGF-I hybrid receptors in skeletal muscle of control subjects and insulinoma patients. Muscle extracts from 12 insulinoma patients (○) and 11 control subjects (●) were added to microwells coated with MA-20. Immobilized receptors were then incubated with ^{125}I-IGF-I (60 pmol/l) in the presence or absence of unlabeled IGF-I (100 nmol/l). Hybrid receptors were estimated as maximal specific IGF-I binding to MA-20–immobilized receptors, and expressed as B/T. Each point represents the mean value for a single subject measured in three experiments carried out in triplicate. The mean for the two groups of subjects is shown as a horizontal bar.

for ^{125}I-IGF-I binding to hybrid receptors, thus indicating that no significant ^{125}I-IGF-I binding to the insulin receptor occurred at the concentration of tracer used. Receptor affinity for IGF-I binding was similar in the two groups of subjects (IC_{50} = 0.59 ± 0.04 and 0.60 ± 0.07 nmol/l IGF-I, for normal subjects and insulinoma patients, respectively). Maximal specific ^{125}I-IGF-I binding to MA-20–immobilized receptors was significantly higher in muscle from insulinoma patients compared with normal subjects (0.57 ± 0.19 vs. 0.36 ± 0.03; $P < 0.0006$) (Fig. 4). The mean values for maximal specific ^{125}I-IGF-I binding for insulinoma patients and normal subjects were 575 ± 190 vs. 372 ± 52 cpm/mg protein. Nonspecific ^{125}I-IGF-I binding determined in the presence of 1 μmol/l unlabeled IGF-I was 11 ± 3% of total ^{125}I-IGF-I binding. Hybrid receptors were also estimated as the fraction of ^{125}I-IGF-I binding immobilized with MA-20 antibody vs. total ^{125}I-IGF-I binding (hybrids + type I receptors) immobilized with α-IGF-IR-PA antibody. The percentage of hybrid receptors was significantly increased in muscle from insulinoma patients compared with normal subjects (71 ± 4 vs. 43 ± 4%; $P < 0.0001$). The abundance of hybrid receptors was inversely correlated with maximal specific insulin binding ($r = -0.61$, $P < 0.0002$). To investigate whether a correlation existed between the interindividual variations in hybrid receptor abundance and fasting plasma insulin levels in insulinoma patients, linear regression analysis was performed. As shown in Fig. 5*A*, the results demonstrated that an abundance of hybrid receptors was positively correlated with fasting insulin levels ($r = 0.82$, $P < 0.0009$). Furthermore, we inquired whether a correlation existed between insulin-mediated glucose uptake and hybrid receptor abundance. As shown in Fig. 5*B*, the amount of hybrid receptors in skeletal muscle of insulinoma patients was negatively correlated with insulin-mediated glucose uptake ($r = -0.8$, $P < 0.01$), whereas it was not correlated with either plasma glucose concentrations ($r = 0.40$, $P = 0.17$) or BMI ($r = 0.1$, $P = 0.77$). Similarly, no correlations were observed between insulin-mediated glucose uptake and maximal specific insulin binding ($r = 0.19$, $P = 0.64$) (Fig. 6).

DISCUSSION

Insulin resistance is a major feature of several disorders, including NIDDM and impaired glucose tolerance (IGT). A number of molecules that are involved in insulin action pathways have been implicated as potential molecular defects responsible for insulin resistance (19). However, investigations into the molecular mechanisms responsible for insulin resistance in patients with NIDDM or IGT have been hampered by difficulties in determining whether defects in insulin action pathways are due to primary abnormalities or to secondary abnormalities in vivo, such as hyperglycemia or hyperinsulinemia. Patients with insulinoma, who are characterized by chronic hyperinsulinemia and insulin resistance (20–22), therefore represent a unique opportunity to discriminate between primary defects and secondary events caused by ambient hyperinsulinemia.

Recently we showed that an abundance of insulin/IGF-I hybrid receptors is increased in skeletal muscle of NIDDM patients compared with that of normal subjects and is correlated with both a decrease in insulin receptor number and an increase in fasting plasma insulin levels (13). This observation, coupled with the accessibility of multiple skeletal muscle biopsies from carefully characterized insulinoma patients, has provided the rationale for studying the role of insulin in regulating hybrid receptor expression. To this end, we quantitated the relative abundance of hybrid receptors in skeletal muscle of normal subjects and insulinoma patients by using a quantitative immunoassay (13), and correlated hybrid receptor abundance with in vivo insulin sensitivity. We found that hybrid receptor abundance is significantly increased in insulinoma patients compared with control subjects. We also found that the increase in plasma insulin levels in insulinoma patients is inversely correlated with insulin receptor number and positively correlated with hybrid recep-

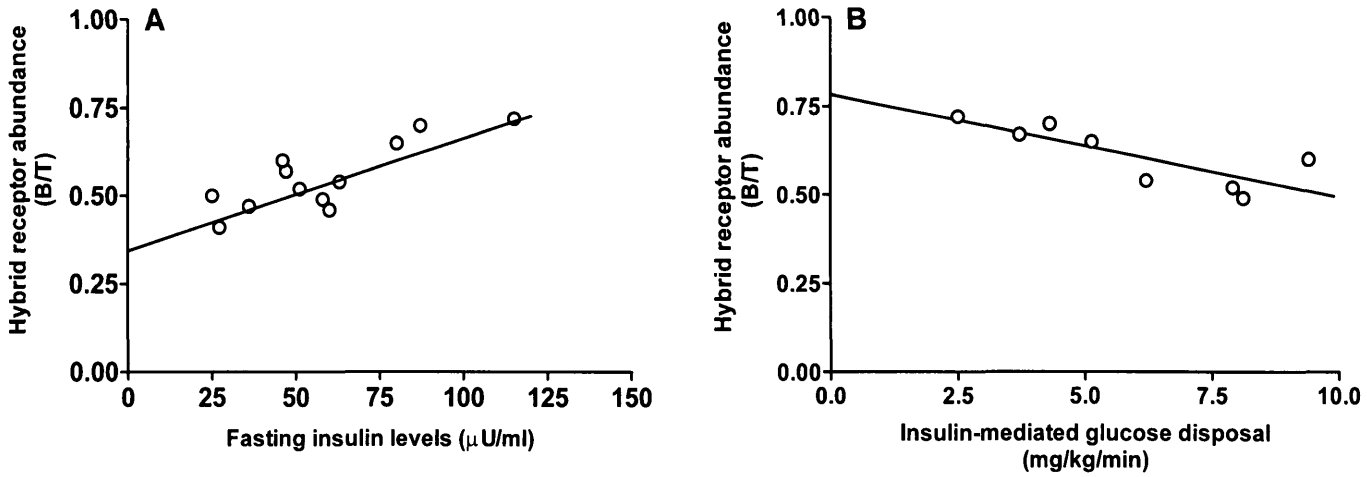

FIG. 5. Abundance of hybrid receptors in skeletal muscle from insulinoma patients expressed as B/T in relation to fasting insulin levels (*A*) and insulin-mediated glucose uptake (*B*).

tor abundance. Because insulin regulates the expression of its own receptor, and assuming that hybrid receptor assembly is proportional to the relative amount of insulin and IGF-IRs in a given tissue, the present findings strongly suggest that a decrease in insulin receptor content due to high plasma insulin levels leads to the formation of a higher proportion of hybrid receptors. Thus it is possible that the defects in hybrid receptor expression observed in NIDDM represent a secondary event due to hyperinsulinemia rather than a primary alteration. Taken together, these data indicate that insulin plays a major role in regulating hybrid receptor assembly.

We also inquired whether the increased abundance of hybrid receptors might contribute, at least in part, to the insulin resistance observed in insulinoma patients. Our findings support this hypothesis, revealing that, in these patients, impaired insulin action is correlated with skeletal muscle hybrid receptor abundance, but not with the amount of insulin receptors. These results are consistent with our previous studies showing a significant correlation between impaired in vivo insulin sensitivity and increased expression of hybrid receptors in muscle of NIDDM patients (13) as well as in placenta of insulin-resistant women with gestational hypertension (23). Overall, these results suggest that alterations in the proportion of hybrid receptors might contribute to impaired insulin action in insulin-resistant subjects by sequestering insulin receptors in a less responsive form. These results also raise the possibility that increased expression of hybrid receptors might represent a generalized defect associated with different states of insulin resistance.

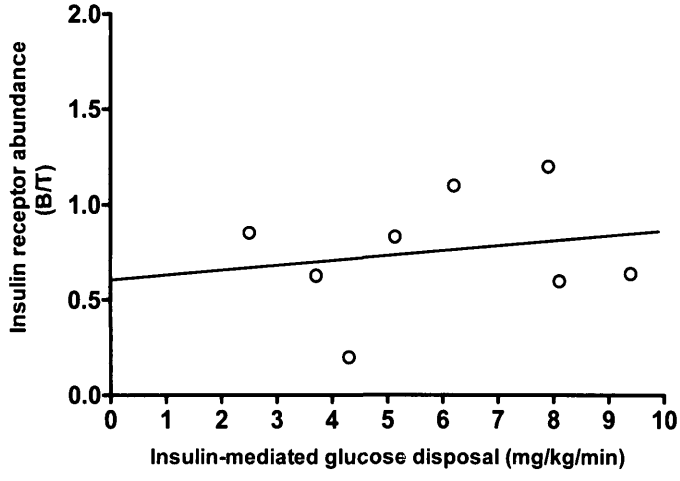

FIG. 6. Maximal specific insulin binding to skeletal muscle extracts from insulinoma patients expressed as B/T in relation to insulin-mediated glucose uptake.

ACKNOWLEDGMENTS

This work was supported in part by grants from BIOMED 2 EC-Programme No. ERB BMH4-CT96-0751 (G.S.) and Consiglio Nazionale delle Ricerche 96.03724.CT14 (G.S.).

We are grateful to Prof. Renato Lauro (Rome, Italy) for his advice and helpful discussions.

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